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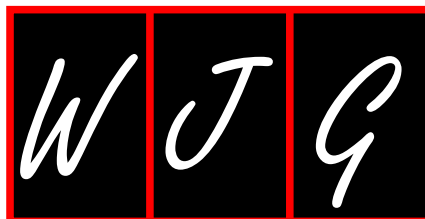
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WJG 20th Anniversary Special Issues (1): Hepatocellular carcinoma

New approaches for precise response evaluation in hepatocellular carcinoma

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Core tip: Accurate tumor burden assessment is a critical component of patient management and the investigation of new therapies. With the increasing clinical use of novel biologic targeted agents or locoregional therapies, morphological analysis confronted limitations, and new methods to assess tumor burden were desired. Advances in imaging technique enable us to assess tumor functions such as viability, vascular physiology, or metabolism, which can be new approaches to assess tumor burden.

Abstract

With the increasing clinical use of cytostatic and novel biologic targeted agents, conventional morphologic tumor burden assessments, including World Health Organization criteria and Response Evaluation Criteria in Solid Tumors, are confronting limitations because of their difficulties in distinguishing viable tumor from necrotic or fibrotic tissue. Therefore, the investigation for reliable quantitative biomarkers of therapeutic response such as metabolic imaging or functional imaging has been desired. In this review, we will discuss the conventional and new approaches to assess tumor burden. Since targeted therapy or locoregional therapies can induce biological changes much earlier than morphological changes, these functional tumor burden analyses are very promising. However, some of them have not gone through all steps for standardization and validation. Nevertheless, these new techniques and criteria will play an important role in the cancer management, and provide each patient more tailored therapy.

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INTRODUCTION

Accurate assessment of tumor burden is an important component of cancer patient management and the investigation of new therapies. Traditionally, therapeutic response has been assessed by serial tumor size measurements according to World Health Organization (WHO) criteria or Response Evaluation Criteria in Solid Tumor (RECIST)^[1-3]. These criteria, which are based on anatomical measurement, are well established tool, and easy to

apply for assessment of tumor burden. However, these morphological evaluations have substantial limitations, including the presence of tumors that cannot be measured, poor measurement reproducibility and mass lesions of unknown activity that persist following therapy^[3]. They also have a difficulty in distinguishing viable tumor from necrotic or fibrotic tissue and recognizing the delay between cell kill and tumor shrinkage. Faced with these limitations, more sophisticated measurements (including tumor volume and lesion regression rates) have been applied to the evaluation of the tumor response to therapy.

With the increasing clinical use of cytostatic and novel biologic targeted agents or locoregional therapies (LRTs) such as ablation and transarterial chemoembolisation (TACE) in the management of hepatocellular carcinoma (HCC), it has become increasingly recognized that new methods of therapy assessment need to be developed urgently. For example, antiangiogenic agents are known to rapidly decrease contrast enhancement on computed tomography (CT)/magnetic resonance imaging (MRI) scans that occur within days of initiation of reduced vascular permeability to contrast agents rather than a true antitumor effect^[4]. Faced with these limitations, the investigation for reliable quantitative biomarkers to assess tumor burden and therapeutic response including blood surrogate parameters, metabolic imaging and functional imaging based on CT, MRI, or positron emission tomography (PET) has been desired^[4-7]. In this review, we discuss various conventional and new approaches to determine tumor burden in the current clinical practice of HCC.

MORPHOLOGIC TUMOR BURDEN ANALYSIS

In 1981, the WHO first published tumor response criteria, mainly for use in trials where tumor response was the primary endpoint. The WHO criteria introduced the concept of an overall assessment of tumor burden by summing the products of bidimensional lesion measurements and determined response to therapy by evaluation of change from baseline while on treatment. Subsequently, RECIST was introduced and approved for clinical use in 2000^[1]. RECIST criteria were primarily conceived to provide specific guidelines for tumor burden measurement. After extensive experience and validation in several chemotherapeutic trials in solid tumors, it was revised in 2009 as RECIST 1.1^[8]. RECIST 1.1 relies on the measurement of a maximum of five target lesions, not exceeding two per organ; subsequently, the sum of the greatest diameters is recorded followed by a final classification^[3]. On the other hand, it has been questioned if these unidimensional measurements can reflect total tumor burden accurately. With the advent of imaging technologies such as workstation and 3D software, longitudinal or oblique measurements readily can be determined, and tumor volumes can be computed algorithmically. Sohaib *et al.*^[9] reported that CT volumetric measurements

were accurate and reproducible in their phantom study. Welsh *et al.*^[10] reported that RECIST might overestimate tumor burden compared with volumetric measurements in HCC and pancreatic cancer, and they concluded that volumetric analysis might be the preferred method to detect tumor progression. However, the practical clinical value of tumor volumetric analysis remains controversial. There is no consensus about the recommended volume equivalents converted from diameter thresholds, which can be effectively applied without sacrificing either reproducibility or sensitivity to tumor progression or partial response.

TUMOR BURDEN ANALYSIS ACCORDING TO VIABILITY AND DENSITY

Recent studies have demonstrated poor correlations between the clinical benefit provided by targeted therapy agents or LRTs and conventional morphologic tumor burden analysis^[11-14]. Unlike cytotoxic agents that may induce rapid tumor shrinkage, targeted therapy agents are acknowledged to yield sustained tumor stabilization and delay tumor progression. For example, antiangiogenic agents can reduce tumor vascularization, provoke areas of necrosis, and sometimes cause cavitation in solid tumors. These peculiar features have been reported with bevacizumab, sorafenib, and sunitinib in HCC^[11,15-18]. In addition, the main objective of all effective LRTs is to induce necrosis of the tumor regardless of the shrinkage of the lesion. Therefore, in 2000, a panel of experts on HCC of the European Association for the Study of Liver (EASL) amended the response criteria to take into account tumor necrosis induced by treatment^[19]. In 2008, The American Association for the Study of Liver Disease developed a set of guidelines that included a formal modification of the response assessment based on the RECIST criteria and aimed to translate into the concept of viable tumor (tumoral tissue showing arterial uptake in the arterial phase of the contrast-enhanced imaging techniques), which are referred to as modified RECIST (mRECIST) criteria (Figure 1)^[20,21]. These criteria are summarized in Table 1. Forner *et al.*^[13] reported that overall response rates of 21.8% for RECIST criteria and 81.8% for EASL in 55 HCC patients treated with a variety of LRTs. Similar findings about overall response rates were reported by Keppke *et al.*^[22] (RECIST 23%, WHO 26%, and necrotic area 59%), Riaz *et al.*^[23] (RECIST 42.4%, WHO 42.4%, and EASL 70.2%), and Prajapati *et al.*^[24] (RECIST 10.8%, WHO 4.1%, EASL 39.2%, and mRECIST 52.5%).

A question then arises which response criteria have the strong association with survival. Previous reports have shown that WHO, RECIST, and EASL responses are associated with improved survival^[23,25], but these studies didn't make the comparison at a single time point. In the phase II study of brivanib in advanced HCC, mRECIST was able to demonstrate a higher response and disease control rate and longer time to progression

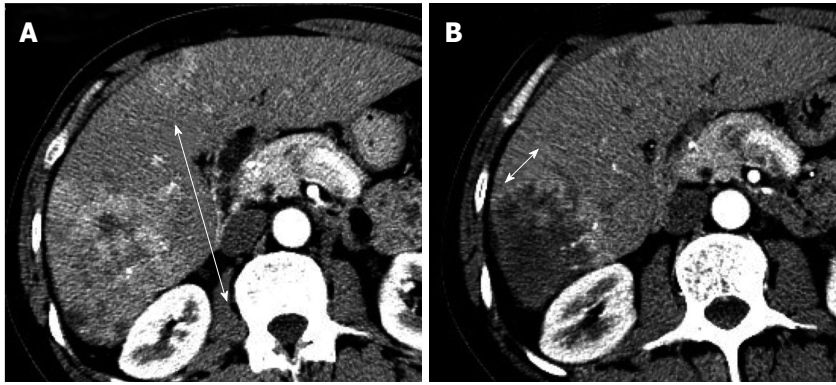


Figure 1 Application of modified Response Evaluation Criteria in Solid Tumor evaluation for hepatocellular carcinoma. A: Baseline; B: Post treatment. Tumor burden change was assessed on arterial-phase contrast enhanced diagnostic computed tomography image. Modified Response Evaluation Criteria in Solid Tumor evaluation should draw the maximal dimension of continuous arterial enhancement in such lesions with central necrosis, avoiding central necrosis.

Table 1 Summary of response criteria

	WHO	RECIST 1.1	EASL	mRECIST
Complete response (CR)	Disappearance of all lesions	Disappearance of all lesions and pathologic lymph nodes	Disappearance of intratumoral areterial enhancement	Disappearance of all lesions and pathologic lymph nodes
Partial response (PR)	$\geq 50\%$ decrease in the sum of the area (longest diameters multiplied by longest perpendicular diameters)	$\geq 30\%$ decrease in the sum of the longest diameters	$\geq 50\%$ decrease in the sum of the arterial enhancing areas (longest diameters multiplied by longest perpendicular diameters)	At least a 30% decrease in the sum of diameters viable (enhancing) target lesions, taking as reference the baseline sum of the target lesions
Stable disease (SD)	Neither PR nor PD	Neither PR nor PD	Neither PR nor PD	Neither PR nor PD
Progressive disease (PD)	$\geq 25\%$ increase in the sum of the area	$\geq 20\%$ increase in the sum of the longest diameters and ≥ 5 mm absolute increase in the sum of the longest diameters	$\geq 25\%$ increase in the size of the arterial enhancing areas or development of a new lesions	$\geq 20\%$ increase in the sum of diameters of viable target lesions recorded since treatment started or development of new lesions

WHO: World Health Organization; EASL: European Association for the Study of Liver; mRECIST: Modified Response Evaluation Criteria in Solid Tumors.

than the WHO criteria^[26]. In a recent retrospective study of HCC patients treated with sorafenib, patients categorized as responder according to mRECIST had a longer overall survival (OS) than non-responder^[27]. Prajapati *et al.*^[24] reported that mRECIST and EASL had significant correlation with survival, whereas WHO and RECIST 1.1 had poor correlation. Another key issue is that radiological assessments with EASL and mRECIST can be carried out at an early time point, in comparison with WHO and RECIST^[12,22,23]. Therefore, response evaluation based on the concept of viable tumor may be valuable for making early decisions regarding further therapy.

The tumor density analysis based on contrast enhanced CT attenuation measurement can serve as an additional method for response assessment in solid tumors^[28]. Choi *et al.*^[28] reported that gastrointestinal stromal tumors treated with imatinib mesylate, reduced tumor density on the portal venous phase CT, which had a correlation with the tumor necrosis, or cystic or myxoid degeneration without changes in tumor size. The tumor density is measured by drawing a region of interest (ROI) circumscribing the boundary of the tumor in the portal venous phase^[29]. In gastrointestinal stromal tumors, a de-

crease in tumor Housefield units $> 15\%$ correlated with progression free survival^[30]. In HCC, a recent studies showed that tumor density measurement on the portal venous phase CT images was more sensitive than RECIST in detecting patients with longer time to progression after sunitinib therapy (Figure 2)^[31].

PERFUSION ANALYSIS

As discussed earlier, the morphologic tumor burden assessment has a difficulty in distinguishing viable tumor from necrotic or fibrotic tissue because molecular targeted agents suppress tumor growth by downregulating angiogenesis without causing much morphologic change. In this sense, the investigation for reliable quantitative assessment of therapeutic response including blood surrogate parameters, metabolic imaging and functional imaging has been desired^[4,5]. Perfusion technique, which enables quantification of tumor vascularity by measuring the temporal changes in tissue density following intravenous contrast administration, are readily incorporated into the existing CT and MRI protocols that continue to provide the mainstay for anatomical imaging in oncol-

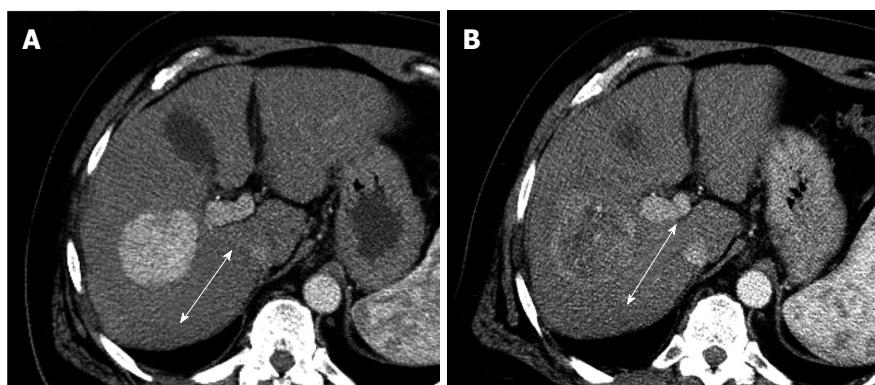


Figure 2 Portal-phase contrast enhanced diagnostic computed tomography of 58-year-old man with hepatocellular carcinoma. A: Baseline; B: Post treatment. Obvious tumor density change was observed after antiangiogenic treatment.

ogy^[32]. Most scanners now come equipped with sophisticated hardware platforms coupled with powerful and user-friendly software packages for tissue perfusion analysis. Perfusion parameters are dependent on the scan protocol and the mathematical model for perfusion analysis^[33,34], but the commonly described perfusion CT parameters include blood flow (BF), blood volume (BV), mean transit time (MTT), and permeability surface area product^[15,16,34]. Similarly for dynamic contrast-enhanced (DCE)-MRI, transfer constant (K_{trans}) is the most accepted quantitative surrogate end point from compartment models^[35-38]. Several studies have demonstrated the value of perfusion imaging for monitoring the effect of antiangiogenic agents advocating various imaging tools in various solid tumors^[14-17,39-43]. Several papers reported that BF or BV decreased even after 2 wk of antiangiogenic therapy (Figure 3)^[15,16]. Moreover, perfusion imaging has a potential to be a biomarker of antiangiogenic therapy^[14,16,41-43]. In perfusion CT, Jiang *et al.*^[16] demonstrated that HCC with higher baseline MTT correlated with favorable clinical outcome. In DCE-MRI study of renal cell carcinoma, high baseline K_{trans} and reduction in K_{trans} after treatment were related to progression free survival (PFS)^[41,42]. In advanced HCC, DCE-MRI demonstrated reduction in K_{trans} during antiangiogenic treatment and the change of K_{trans} during treatment was related to better PFS and OS in clinical trials of tyrosine kinase inhibitors^[14,17,43].

Considering the accessibility and availability, Perfusion CT is superior to DCE-MRI. However, relatively high radiation dose and limited coverage of the anatomy are two major drawbacks of perfusion CT. Therefore, several efforts are being made with low dose scanning technique^[34]. In addition, there is no consensus on a scanning protocol or a mathematical model in abdominal lesion. The definition of the tumor ROI and the acquisition time is also a subject to similar consideration^[44,45].

On the other hand, DCE-MRI has the advantage of lack of ionizing radiation, good spatial resolution and soft-tissue contrast. However, it is one of the most expensive and still technically challenging imaging modalities, requiring longer image acquisition times and provides smaller interscan reproducibility, as compared with

CT^[46,47]. DCE-MRI also lacks the standard protocol and the established response evaluation criteria.

Regardless of these limitations, perfusion technique must be a potentially powerful tool for HCC patient management, which may enable prediction or early detection of therapy responder.

DIFFUSION-WEIGHTED MRI

Molecular diffusion, or Brownian motion, was first formally described by Einstein^[48] in 1905. Various tissue types have unique diffusion characteristics, as measured by the apparent diffusion coefficient (ADC), which can be calculated by the diffusion-weighted imaging (DWI) measurements acquired with a different gradient duration and amplitude (*b*-values). The movement of water molecules in biological tissues within the body is typically limited by interactions with cell membranes macromolecules, and fibers in tissue compartments. Therefore, DWI has been suggested as a tool to distinguish different tissue compartments and detect changes in cellular tissue structures and viability, which could be used to monitor the response to treatment. DWI has been discussed as cancer biomarker in a consensus meeting and a publication on consensus and recommendations for DWI as a cancer biomarker has been published recently highlighting the potential of this promising technique in cancer patients^[49]. In lung cancer, a previous study reported that ADC values differ based on histological type, which suggested a possible correlation between ADC values and tumor characteristics, such as histology, response to therapies and prognosis^[50]. Monitoring effectiveness of treatment is often challenging, especially following liver directed therapy. In HCC, the usefulness of DWI in the evaluation of therapeutic efficacy after targeted therapy or TACE has already been reported in several studies^[51-55]. Some of those studies reported that the ADC value in HCC showed significant increases after TACE^[51-53]. Yuan *et al.*^[55] reported that high baseline ADC value of HCC could predict poor response to TACE and that responding lesions had a significant increase in %ADC values than nonresponding during TACE. They demonstrated

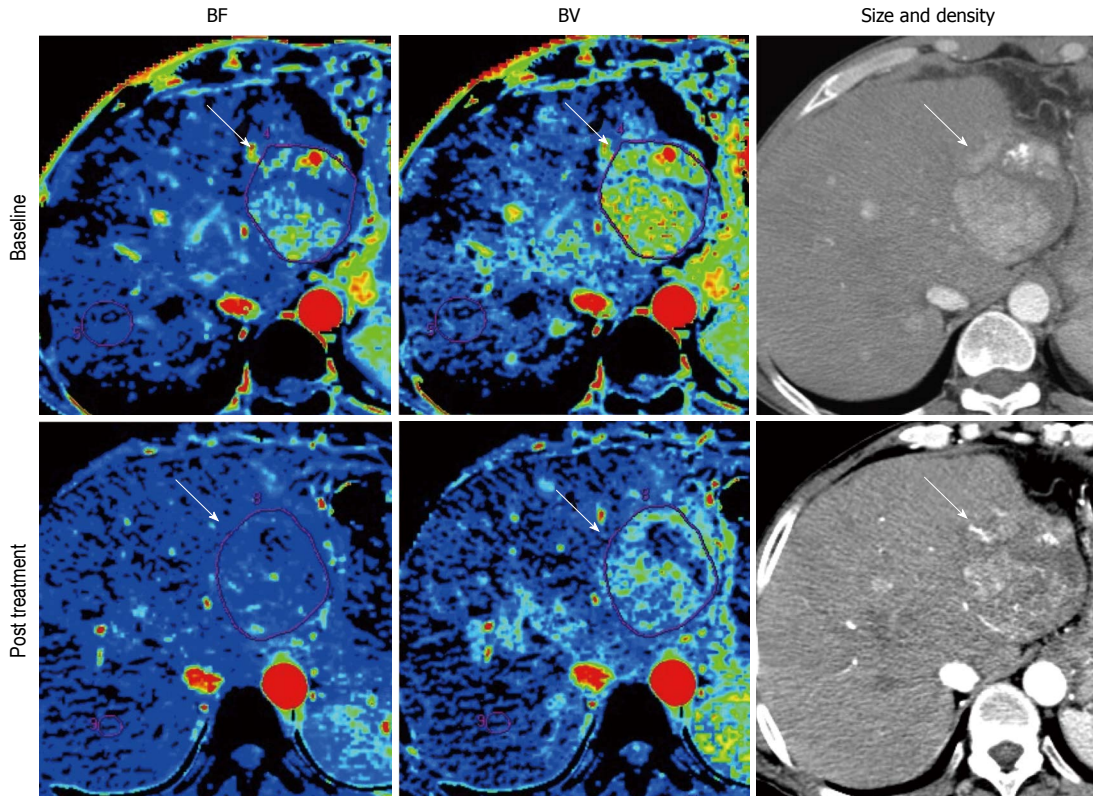


Figure 3 Perfusion maps of 53-year-old man with hepatocellular carcinoma. Parameters measure by perfusion computed tomography showed substantial changes in comparison with tumor size and density at only 2 wk after antiangiogenic treatment. Blood flow (BF), blood volume (BV) were -75.5% and -59.5%. On the other hand, those of size and density were not so obvious (-3.0% and -18.1%).

that an alteration %ADC value $\geq 16.21\%$ could be used to identify HCC to early response to chemoembolization. In HCC treated with an antiangiogenic agent (sorafenib), Schraml *et al.*^[54] reported that early decrease in ADC of tumor after therapy was followed by an increase (Figure 4). However, there are some limitations regarding ADC values reproducibility, which depend on magnetic field strength, technical factors (*e.g.*, *b*-value selection) and on the ROI localization on ADC maps^[56,57]. In addition, particularly in abdomen, DWI still represents a technical challenge because of the strong influence of motion caused by breathing and vascular pulsation, resulting in image artifacts that may lead to inaccurate ADC measurements^[58]. Nevertheless, DWI is one of the promising techniques for the noninvasive assessment of tumor burden. Future studies are necessary to correlate the time course of ADC changes with HCC therapy response, and additional technical developments are necessary to improve DWI quality and spatial resolution.

PET

PET is a quantitative imaging modality using various tracers such as ^{18}F -fluorodeoxyglucose (^{18}F -FDG)^[59-63], ^{11}C -acetate (^{11}C -Act)^[64-67], ^{11}C - or ^{18}F -F-choline (^{11}C -Cho, ^{18}F -F-Cho)^[68] and ^{18}F -fluorothymidine (^{18}F -FLT)^[69] to assess metabolism, lipogenesis, cellular membrane metabolism and proliferation respectively.

^{18}F -FDG, which can be used for assessing glucose metabolism of tumors, is the most widely available clinical PET tracer (Figure 5). Generally, malignant tumors show increased ^{18}F -FDG uptake due to the increased number of glucose transporters and the increased hexokinase activity. Nevertheless, FDG-PET shows poor sensitivity for the detection of HCC with reports ranging from 50% to 55%^[70-74]. In spite of the poor sensitivity of ^{18}F -FDG PET in HCC, Song *et al.*^[75] reported that the increase of ^{18}F -FDG uptake in HCC was significantly associated with tumor burdens such as size and number of tumors, and they concluded that ^{18}F -FDG PET could provide effective information on the prognosis of the treatment response. In addition, it has been demonstrated that ^{18}F -FDG uptake after TACE might be a favorable marker to assess tumor viability after TACE^[66-76]. Similar findings have been reported in detecting local tumor progression following radiofrequency ablation of HCC^[77].

Despite the rapid integration of PET with ^{18}F -FDG into clinical practice, there has been relatively little systematic integration of PET into clinical trials of new cancer treatments. Given the clinical importance and quantitative nature of PET, it is important to have methods to allow inclusion of PET response criteria into clinical trials. Therefore, the European Organization for Research and Treatment of Cancer (EORTC) has defined response assessment criteria for PET in 1999^[78]. Although some use the EORTC criteria, methods for PET performance and

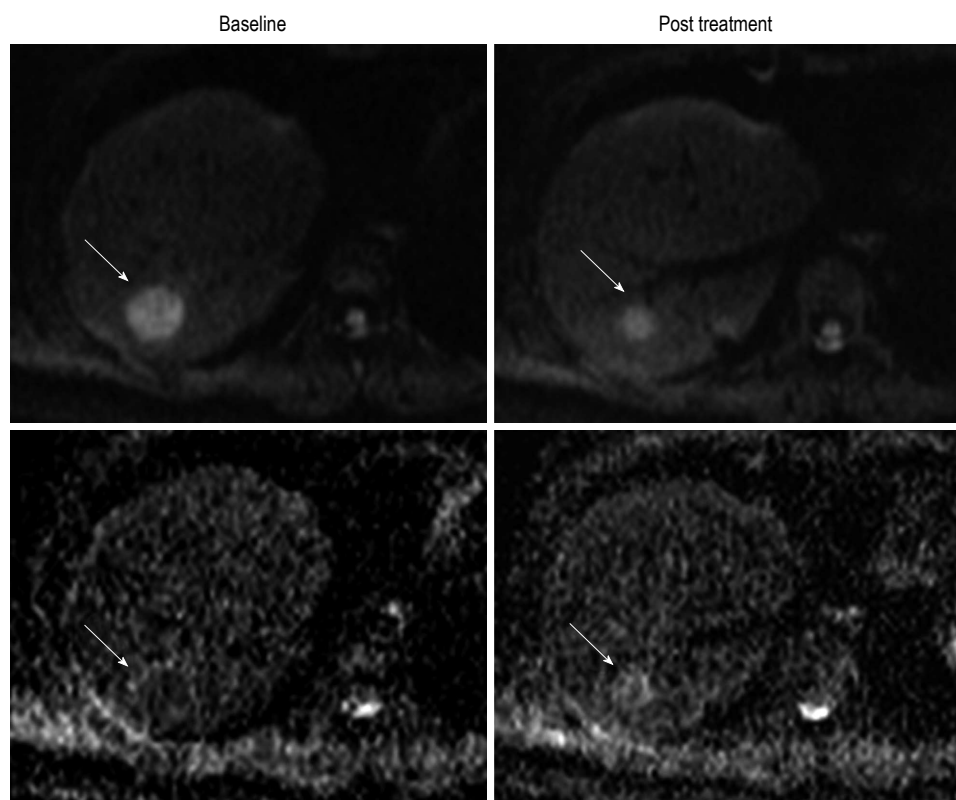


Figure 4 Diffusion-weighted imaging and apparent diffusion coefficient map at baseline and post treatment of 31-year-old woman with hepatocellular carcinoma (arrows). This patient was treated with antiangiogenic agent (sunitinib). Apparent diffusion coefficient showed 18.8% increase (from 1.28×10^{-3} to 1.52×10^{-3} mm²/s) after antiangiogenic treatment.

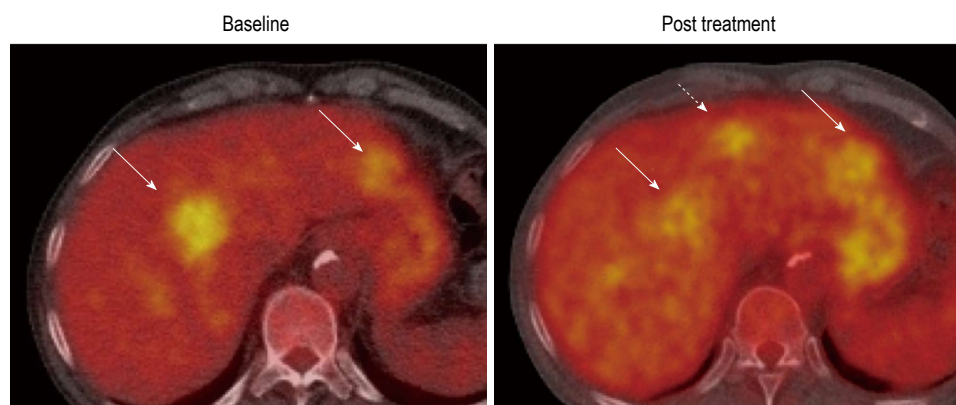


Figure 5 Positron emission tomography/computed tomography of 57-year-old man with hepatocellular carcinoma at baseline and post treatment. He was treated with a systemic chemotherapy. New lesion was detected by a follow-up positron emission tomography/computed tomography (dotted arrow).

interpretation are typically highly variable across studies and typically only exploratory. Therefore, in 2009, Wahl *et al.*^[79] described the Positron Emission Tomography Response Criteria in Solid Tumors (PERCIST) 1.0 to standardize therapy-monitoring method with PET. They classified responses by use of percentage changes in SUVs in the “hottest” lesions per scan. The basics of PERCIST 1.0 are shown in Table 2, where they are contrasted with the EORTC criteria. It is clear that further efforts are needed to validate usefulness of SUV as a sensitive biomarker to assess tumor burden, response and clinical outcome. At

present, PET still plays a small role in imaging assessment of HCC tumor burden, compared with other modalities, but tumor-specific tracers may be the key in future.

CONCLUSION

Accurate tumor burden assessment is a critical component of patient management and the investigation of new therapies. Morphological tumor burden analysis has been served as golden standard. However, with the increasing clinical use of novel biologic targeted agents or LRTs,

Table 2 Comparison of European Organization for Research and Treatment of Cancer and Positron Emission Tomography Response Criteria in Solid Tumors 1.1

	EORTC	PERCIST
CMR	Complete resolution of ^{18}F -FDG uptake within the tumor volume so that it is indistinguishable from surrounding normal tissue	Complete resolution of ^{18}F -FDG uptake within measurable target lesion so that the liver activity was less than the mean and indistinguishable from surrounding background blood-pool levels plus disappearance of all other lesions to background blood-pool levels and appearance of no new ^{18}F -FDG-avid lesions
PMR	Minimum 15%-25% reduction in tumor ^{18}F -FDG SUV after 1 chemotherapy cycle and > 25% reduction after \geq 1 treatment cycle; reduction in extent of tumor ^{18}F -FDG uptake not required	\geq 30% relative and \geq 0.8 SUL unit absolute reduction in target measurable tumor ^{18}F -FDG SUL peak and no increase > 30% in SUL or size (per RECIST) of target or nontarget lesions or appearance of new lesions; reduction in extent of tumor ^{18}F -FDG uptake not required \geq 30% decrease in the sum of the longest diameters
SMD	< 25% increase or < 15% decrease in tumor ^{18}F -FDG SUV and no visible increase in extent of ^{18}F -FDG tumor uptake (> 20% in the longest dimension)	Not CMR, PMR, nor PMD
PMD	> 25% increase in ^{18}F -FDG tumor SUV within the tumor region defined on the baseline examination or visible increase in the extent of ^{18}F -FDG tumor uptake (> 20% in the longest dimension) or appearance of new ^{18}F -FDG uptake in metastatic lesions	> 30% increase in ^{18}F -FDG SUL peak, with > 0.8 SUL unit increase in tumor SUV peak from baseline scan in pattern typical of tumor and not of infection/treatment effect or visible increase in extent of ^{18}F -FDG tumor uptake (75% in total lesion glycolysis volume with no decline in SUL) or new ^{18}F -FDG-avid lesions typical of cancer and not related to treatment effect or infection

CMR: Complete metabolic response; PMR: Partial metabolic response; SMD: Stable metabolic disease; PMD: Progressive metabolic disease; EORTC: European Organization for Research and Treatment of Cancer; PERCIST: Positron Emission Tomography Response Criteria in Solid Tumors; FDG: Fluorodeoxyglucose; SUV: Standardized uptake values.

morphological analysis confronted limitations, and new methods to assess tumor burden were desired. Advances in software and hardware of imaging technique enable us to assess tumor function such as viability, vascular physiology, or metabolism. Since targeted therapy or LRTs can induce biological changes much earlier than morphological changes, these functional tumor burden analyses are very promising. However, some of them have not gone thorough all steps for standardization and validation. Nevertheless, these new techniques and criteria will play an important role in the cancer management, and provide each patient more tailored therapy.

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WJG 20th Anniversary Special Issues (1): Hepatocellular carcinoma

Transarterial chemoembolization and bland embolization for hepatocellular carcinoma

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Transarterial embolization; Prognosis

Core tip: In the current article, we review the use of transarterial chemoembolization (TACE) and transarterial embolization (TAE) for hepatocellular carcinoma and we focus on the evidence for their use. Apart from their use in intermediate stage hepatocellular carcinoma, we also review the evidence for their use as neo-adjuvant treatment in the pre-transplant setting. We also highlight the fact that there is no conclusive evidence for superiority of TACE over TAE.

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Abstract

Transarterial chemoembolization (TACE) is the first line treatment for patients with intermediate stage hepatocellular carcinoma but is also increasingly being used for patients on the transplant waiting list to prevent further tumor growth. Despite its widespread use, TACE remains an unstandardized procedure, with variation in type and size of embolizing particles, type and dose of chemotherapy and interval between therapies. Existing evidence from randomized controlled trials suggest that bland transarterial embolization (TAE) has the same efficacy with TACE. In the current article, we review the use of TACE and TAE for hepatocellular carcinoma and we focus on the evidence for their use.

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Key words: Cirrhosis; Hepatocellular carcinoma; Mortality; Embolization; Transarterial chemoembolization;

INTRODUCTION

Hepatocellular carcinoma (HCC) is the 6th most common cancer worldwide with over 500000 new cases diagnosed each year and the third most common cause of cancer-related death^[1]. Its incidence in Europe is 3.6/100000-10.6/100000 persons^[2] and rises to 16/100000 persons worldwide^[3].

Mortality rates remain high and only 5% of patients survive at 5 years after diagnosis; this is largely due to the fact that diagnosis is most often delayed, with only 15% of patients eligible for surgical procedures such as resection and liver transplantation, 50% for non-surgical therapies and 35% or more for best supportive care at diagnosis^[3]. Both the American (AASLD)^[4] and European (EASL) Associations for the Study of the Liver^[5] have recently published updated guidelines for the management of HCC. These are based on a stratification of patients according to the Barcelona Clinic Liver Cancer (BCLC)

classification, which classifies patients according to tumor burden, liver function as assessed by Child-Pugh score, and performance status, into five distinct prognostic categories with different first line treatment recommendations^[6].

Intermediate stage HCC or stage B according to BCLC, consists of multi-nodular tumors in patients with Child-Pugh A or B cirrhosis and good performance status^[7]. The recommended first line treatment for these patients is transarterial chemoembolization (TACE)^[8]. It should be noted that patients with intermediate stage HCC are considered a heterogeneous group with different prognoses and responses to treatment leading to both European and Asian experts identifying the need for further sub-classification^[9,10]. This could be based on tumor size and Child-Pugh score^[10]. Indeed, in carefully selected patients with preserved liver function intermediate HCCs, hepatic resection could be a more effective therapeutic option than TACE^[11]. The future use of molecular signatures and markers might further enhance the classification and prognosis^[12].

In the current article, we review the use of transarterial embolization (TAE) with (TACE) or without (TAE) the use of chemotherapeutic agents for HCC focusing on the evidence for their use.

TACE AND TAE

The normal liver receives a dual blood supply from the hepatic artery (25%) and the portal vein (75%). As HCC grows, it increasingly depends on the hepatic artery for blood supply and once a tumor nodule reaches a diameter of 2 cm or more, most of the blood supply derives from the hepatic artery. This unique property of HCC provides the rationale for the use of transarterial therapies. TACE and TAE consist of the selective angiographic occlusion of the tumor arterial blood supply with a variety of embolizing agents, with or without the precedence of local chemotherapy infusion. The occlusion by embolic particles results in tumour hypoxia and necrosis, while the addition of local chemotherapy could have an additive anti-tumour effect. The efficacy of TA(C)E was established by a meta-analysis published a decade ago that included 6 randomized controlled trials (RCTs), of which only two were positive, and showed improved two-year survival (HR = 0.53; 95%CI: 0.32-0.89; $P = 0.017$)^[13]. Patients included in those trials were not staged according to BCLC, as this was not available at that time, and selection criteria differed from current recommendations. More recently our updated meta-analysis, which included nine RCTs also demonstrated a survival benefit of TA(C)E compared to best supportive treatment (HR = 0.705; 95%CI: 0.5-0.99)^[14]. A recent Cochrane meta-analysis, including nine RCTs with 645 participants, failed to demonstrate a survival benefit with TA(C)E *vs* best supportive care (HR = 0.88; 95%CI: 0.71-1.10) and concluded that an additional 383 participants would need randomization for a potential benefit to be demonstrated^[15]. This meta-

analysis was heavily criticized for the exclusion of positive RCTs due to risk of bias and inappropriate inclusion of trials using gelfoam with short follow-up or enrolling patients with early stage HCC^[16]. The inconsistency in the results of randomized trials reflects the fact the TA(C)E is not a standardized procedure both in terms of patient selection and the procedure itself.

PATIENT SELECTION AND SURVIVAL

According to current treatment guidelines, TA(C)E is not a curative treatment and should be considered as first line treatment in patients with intermediate HCC. In clinical practice however TACE is frequently used outside these recommendations in a wide range of patients ranging from early HCC to advanced liver disease with ascites. Therefore, the variation in reported survival is likely to be dependent on patient selection as well as TA(C)E schedule and techniques.

A retrospective Italian study compared the results of TACE before and after the implementation of BCLC criteria in 2002; In the 1999-2002 period, there was no significant difference in survival between TACE-treated and untreated patients, while in the 2003-2006 period, TACE-treated patients survived longer ($P < 0.0001$) following the significant increase in Child Pugh class A patients and advanced HCC^[17]. Another retrospective Italian study, including 614 elderly and 1104 younger patients with HCC, showed that overall applicability of HCC treatments was unaffected by older age although treatment distribution differed, with elderly individuals being more frequently treated with percutaneous procedures and less frequently with resection or TACE^[18]. A retrospective Chinese study that included 1516 patients with HBV-related cirrhosis and BCLC stage B HCC, all of who received TACE as first line treatment, reported 1-, 3- and 5-year overall survival rates of 84%, 29% and 19% respectively^[19]. Child-Pugh A liver function and smaller tumor were associated with treatment response. Tumor response after initial TACE, an independent prognostic factor of overall survival, was associated with tumor extent and influenced subsequent treatment^[19].

A Spanish cohort study of a highly selected patients, 40% of whom were staged as BCLC-A, reported a median survival of 48.6 mo following TACE with pre-loaded drug eluting beads (DEB-TACE) after a median follow up of 24.5 mo^[20]. Unfortunately, this conclusion was based on projected and not actual data, as at the time point of median survival, less than 15 patients were still at risk of death according to the Kaplan-Meier curve and 35 had died, *i.e.*, there was no adequate follow up for at least 50% of the cohort^[21]. A Japanese retrospective study reported the outcomes of TACE in 4966 patients diagnosed between 2000 and 2005 across all spectrums of Child Pugh classes and tumor size; overall median and 5-year survivals were 3.3 years and 34%, respectively^[22]. Not surprisingly, the study showed that the survival rate decreased as the tumor number and size increased in all

but one subgroup in both Child-Pugh-A and -B^[22].

TA(C)E TECHNIQUES

A systematic review of 175 cohort and randomized trials of transarterial therapies for HCC exposed the huge heterogeneity in the protocols used, with variable use of embolic and chemotherapeutic agents, variable embolization particle sizes, different schedules and indications for repeat sessions and different arterial selectivity for embolization^[23].

EMBOLIZING AGENTS

Over the years, a variety of embolizing agents have been used, from gelfoam to polyvinyl alcohol (PVA) particles and more recently drug eluting beads.

Gelfoam, which consists of gelatin sponge particles, was used in the first trials of TACE and is a suboptimal embolizing agent, due to the large size of the particles (1 mm) and the temporary occlusion of the tumor feeding arteries that only lasts for 2 wk^[24,25].

PVA particles provide more permanent arterial occlusion and can potentially provide more distal arterial obstruction as their size can be as small as 45-150 microns^[25]. A recent study with histopathological analysis of embolized tumors confirmed that smaller PVA particles can reach and occlude more distal arteriolar capillaries and result in slightly better tumor necrosis rate after TACE^[26]. Moreover, a non-randomized trial comparing different embolizing agents, demonstrated that the number of TACE sessions was significantly greater for the gelfoam powder group (mean, 2.2) *vs* the PVA group (mean, 1.6; *P* = 0.01), although survival did not significantly differ^[27].

Drug-eluting beads (DEBs; Biocompatibles, Surrey, United Kingdom) is a novel system consisting of embolic microspheres preloaded with doxorubicin, that ensure the controlled release of chemotherapy and thus provide a combined local ischaemic and cytotoxic effect^[28]. This results in lower systemic doxorubicin concentrations than conventional TACE and higher intra-tumor retention^[28]. However, a phase II RCT comparing DEB-TACE with conventional TACE failed to demonstrate a superiority of DEB-TACE in tumor response^[29]. Drug related adverse events and liver toxicity were lower in the subgroup of patients with Child Pugh class B and bi-lobe tumors, allowing better adherence to treatment protocol and higher objective response rates in this particular subgroup^[29]. It should be noted that conventional TACE was not standardized and a variety of embolizing particles and treatment schedules were used, according to the preferences of the treating physician. A RCT comparing TACE with DEBs and TAE with the same particles but without chemotherapy, failed to demonstrate any significant differences in survival or tumor response, further questioning the efficacy of the preloaded chemotherapy^[30]. DEBs are more expensive than conventional TA(C)E with as yet unproven superiority. However they do represent an

important step towards the standardization of the technique and might increase tolerability in sicker patients^[7].

CHEMOTHERAPEUTIC AGENTS

Doxorubicin and cisplatin, followed by epirubicin, are the most commonly used chemotherapeutic agents but none has proven superior to date^[23] and the choice usually relies on local protocols and physicians preferences. The dosing of chemotherapeutic agent also varies among centres. The median dose in published trials per session of doxorubicin, cisplatin and epirubicin was 50 mg, 92 mg and 50 mg respectively^[23]. There is no consensus if a standard dose for all patients should be used or a dose adjusted to the body surface area or whether the bilirubin level or other measure of liver function is preferable. As already mentioned, patients with more advanced liver disease might benefit from DEB-TACE due to lower systemic chemotherapy concentrations. Higher chemotherapeutic doses did not significantly enhance the anticancer effects and survival compared that with lower doses in a study published a decade ago^[31].

FREQUENCY OF TA(C)E SESSIONS

The frequency of TA(C)E has not been adequately addressed to date. From an oncological point of view, chemotherapy should be administered at 3-week intervals in order to fit to the cell cycle^[8]. However, such a strategy would carry the risk of increased side effects and indeed patients with an initial good response would not necessarily benefit. A repeat "on demand" strategy of conventional TACE was recently retrospectively evaluated in 151 consecutive patients. Complete response and recurrence rates after first and second TACE were similar, with 64% of patients being submitted to second TACE and 26% to third TACE using an "on demand" policy^[32] based on tumor response. We have reported the case of a patient with 10 "on demand" TAE over a 5-year period with repeated radiological response^[33].

ADVERSE EFFECTS

The most common adverse effect of TA(C)E is the post-embolization syndrome, which is manifested by abdominal pain, fever and elevated liver function tests in the first 24-48 h post-treatment and only requires supportive measures^[34]. Deterioration of liver function with development of ascites and even liver failure occurs in a minority of patients and depends on liver reserve pre-TACE and selectivity of embolisation. Our systematic review reported a median treatment related mortality of 2.4% in 37 trials including 2878 patients^[23], however this is influenced by patient selection. A recent Japanese cohort study reported treatment related mortality of 0.38% (19/4966 patients)^[22]. However, in the presence of ascites, 17% of patients with develop liver failure post-TACE and the vast majority of them will die within a year^[35]. Other TACE-induced adverse events include the formation of

Table 1 Randomized controlled trials comparing transarterial embolization with transarterial chemoembolization in patients with hepatocellular carcinoma

Ref.	Patients, <i>n</i>	Chemotherapy in TACE arm	Embolizing agent	Outcome in survival
Kawai <i>et al</i> ^[41] , 1992	289	Doxorubicin	Lipiodol + gelfoam	NS
Chang <i>et al</i> ^[42] , 1994	46	Cisplatin	Lipiodol + gelfoam	NS
Llovet <i>et al</i> ^[43] , 2002	77	Doxorubicin	Lipiodol	NS
Malagari <i>et al</i> ^[30] , 2010	84	Doxorubicin-loaded LC beads	BeadBlocks	NS
Meyer <i>et al</i> ^[39] , 2013	86	Cisplatin	PVA particles	NS
Brown <i>et al</i> ^[40] , 2012	101	Doxorubicin-loaded LC beads	BeadBlocks	NS

TACE: Transarterial chemoembolization; NS: Non-significant; PVA: Poly-vinyl alcohol.

liver abscess in the necrotic tumor, bile duct injury and ischaemic cholecystitis^[7].

TAE OR TACE

TACE is reported as the preferred transarterial therapy of choice in the literature^[1,3] and EASL guidelines^[5], despite the fact that this claim is not supported by existing evidence^[36,37]. From a pathophysiological point of view, TACE in most centres consists of the chemotherapy and embolization administered at the same time. As hypoxia is a known cause of chemo-resistance, the rationale for administering chemotherapy while rendering the tumour hypoxic is questionable^[38].

We recently published an updated meta-analysis of five RCTs comparing TACE with TAE, where we found no difference in survival^[39]. Since then, a sixth RCT was published in abstract form, also with unequivocal results^[40]. All these published RCTs are summarized in Table 1^[30,39-43]. The last three RCTs used permanent occluding embolizing particles^[30,39,40], as opposed to gelfoam used in the rest^[41-43].

In the study by Malagari *et al*^[30], 84 patients were randomized to either DEB-TACE or embolization alone using BeadBlocks (100-300 or 300-500 microns diameter). There was no difference in 1-year survival, (86% *vs* 85.3% in DEB-TACE and TAE respectively) despite the fact that the DEB-TACE group had a statistically significant longer time to tumour progression. The RCT performed by our group was a phase II/III trial of three weekly cisplatin based TACE *vs* TAE with PVA particles (diameter 40-150 microns) as the embolizing agent^[39]. The median overall survival and progression-free survival was 17.3 *vs* 16.3 ($P = 0.74$) mo and 7.2 *vs* 7.5 ($P = 0.59$), in the TAE and TACE groups respectively^[39]. Finally, the RCT by Brown published in abstract form, compared DEB-TACE with TAE with BeadBlocks and reported no significant differences in progression-free survival (7 mo *vs* 9 mo) and overall survival (16 mo *vs* 14 mo)^[40].

These data clearly demonstrate that TAE is equally effective as TACE at a lower cost and with potentially fewer side effects due to the lack of chemotherapy^[7]. This lack of additional effect of chemotherapy could be due to the infrequent intervals of TACE of several mo that do not follow the usual oncological chemotherapy principles that target certain cell cycle phases^[44]. They could also be attributed to the chemoresistance that results from tumour hypoxia induced by embolization^[38]. We therefore advocate that the use of permanently occluding embolizing agent is more important than the use of chemotherapy.

ASSESSMENT OF TREATMENT RESPONSE

Traditionally, tumor response was assessed with the RECIST criteria, which are based on the sum of unidimensional measurements of tumor lesions and therefore require tumor shrinkage in order to document response. Transarterial therapies for HCC exert their therapeutic effect by tumor devascularization and necrosis, which is not always accompanied with reduction in size. In order to address this, EASL advocated the measurement of change in tumor enhancement on contrast enhanced imaging (EASL criteria), while AASLD proposed the modified RECIST criteria (mRECIST), that also assess changes in tumor arterial enhancement.

It was recently shown in a cohort of 160 consecutive patients with HCC that evaluating the largest two lesions is generally the most useful procedure for measuring TACE responses under both EASL and mRECIST^[45].

The prognostic implication of treatments response according to mRECIST and EASL compared to RECIST has been assessed in cohort studies. In a cohort of 83 consecutive patients with HCC, we showed that when measured at a single time point after the first transarterial therapy, EASL and mRECIST overall response rates were significantly associated with survival, in contrast with RECIST response rates^[46]; EASL response was associated with a 44% risk reduction and mRECIST with a 42% reduction. These findings were confirmed in a cohort of 114 Korean patients with HCC, where both EASL response (HR = 0.21, 95%CI: 0.11-0.40, $P < 0.001$) and mRECIST response (HR = 0.31, 95%CI: 0.17-0.59, $P < 0.001$) after 1-2 TACE sessions were independently associated with survival. Similarly, the use of mRECIST and EASL response criteria 1 mo after initial TACE, better predicted the differences in overall survival between responders and non-responders than conventional RECIST criteria^[47].

TA(C)E IN PATIENTS WITH PORTAL VEIN THROMBOSIS

PVT is common in patients with cirrhosis and becomes more prevalent as liver function deteriorates^[48]. TACE is generally contra-indicated in patients with PVT, due to concerns that a further decrease to the blood supply of

Table 2 Prognostic scores of survival after transarterial chemoembolization and transarterial embolization

Ref.	Parameters	Cut-off	Comments
Llado <i>et al</i> ^[57]	AFP > 400 ng/mL Tumor volume > 50%	Based on regression co-efficients	Patients classified in 3 categories
Pinato <i>et al</i> ^[58]	Child-Pugh score Neutrophil-to-lymphocyte ratio	Significant improvement in survival if NLR stable or normalized post TACE	Radiological response after TACE also associated with survival
Kadalayil <i>et al</i> ^[59]	Albumin < 36 g/dL bilirubin > 17 µmol/L AFP > 400 ng/mL Dominant tumor > 7 cm	4 groups based on HAP scores of 0, 1, 2 and > 2	Validated in an independent dataset
Sieghart <i>et al</i> ^[60]	Increase of AST > 25% Increase of Child-Pugh > 1 Absence of radiologic tumor response	0-1.5 points; ≥ 2.5 points	Determines prognosis prior to 2 nd TACE; validated in independent cohort

TACE: Transarterial chemoembolization; HAP: Hepatoma arterial-embolization prognostic; NLR: Neutrophil to lymphocyte ratio.

the liver can prove deleterious. Nevertheless, this dogma has been challenged and there are uncontrolled trials and cohort studies that suggest a treatment benefit in selected patients with preserved liver function^[49,50]. A recent meta-analysis including 8 studies with 1601 patients, concluded that TACE in patients with portal vein thrombosis (PVT) improved the 6-mo (HR = 0.41; 95%CI: 0.32-0.53) and 1-year (HR = 0.44; 95%CI: 0.34-0.57) survival compared with conservative treatment^[51]. Nevertheless, studies included in the meta-analysis exhibited significant differences among patient characteristics between the treatment groups, and were ill defined in terms of treatment allocation^[51]. Until these results are confirmed in RCTs, they need to be interpreted with extreme caution and decisions should be made on an individual patient basis taking into account the radiological expertise of the treating centers. We would only consider TA(C)E in patients with Child A cirrhosis and segmental PVT.

COMBINATION OF TA(C)E AND PERCUTANEOUS TECHNIQUES

The effectiveness of percutaneous techniques, mainly represented by radiofrequency ablation (RFA), is reduced as tumour size increases. This is partly due to the increased blood flow in larger lesions resulting in heat loss and thus less effective ablation^[7]. Therefore, it seems reasonable to perform RFA after occluding the hepatic arterial flow supplying the tumour with TA(C)E. This would theoretically increase the ablation size of thermal injury as blood flow to and within the tumour is reduced. To date, there have been no large and conclusive RCT

assessing dual sequential therapy. In a RCT including 93 patients with tumours less than 3 cm, combination treatment with TACE and RFA did not result in improved survival compared to RFA alone^[52], which was a predictable result given the small size of tumours. In another RCT that included 189 patients with tumours < 7 cm, combined RFA and TACE resulted in better overall and tumour free survival than RFA alone^[53]. There are only cohort studies that compare TACE and RFA *vs* TACE alone and these have shown promising results that warrant adequately powered RCTs^[54,55].

PREDICTION OF TREATMENT RESPONSE AND POST-TREATMENT SURVIVAL

Survival in patients with HCC depends on both the successful treatment of the tumor but also on the underlying liver function and reserve^[56]. Therefore, not surprisingly, survival post transarterial therapies is independently influenced by a combination of tumor and liver function parameters (Table 2).

A simple prognostic score consisting of alpha-fetoprotein (> 400 U/L), tumor size (> 50%) and Child-Pugh score was found to predict the survival of patients treated with TACE and could therefore be used to decide which patients with unresectable HCC should receive this therapy; the authors concluded that TACE should not be administered to patients with one or more positive prognostic factors^[57]. Similarly, it was shown that patients with a persistently increased neutrophil-to-lymphocyte ratio post-TACE have a worse outcome^[58]. We recently developed and validated a simple prognostic score for post TA(C)E survival, namely Hepatoma arterial-embolisation prognostic (HAP) score, where one point is assigned for each of albumin < 36 g/dL, bilirubin > 17 µmol/L, AFP > 400 ng/mL or size of dominant tumour > 7 cm^[59]. This is simpler than previously described scores^[57] and only requires calculation at a single time point rather than serial measurements^[58].

Similarly, the Assessment for Retreatment with TACE (ART) score was developed and validated in order to guide the decision for retreatment with TACE^[60]. The increase of AST by > 25%, an increase of Child-Pugh score of 1 (or ≥ 2 points) from baseline, and the absence of radiologic tumor response were used to create the ART score. The ART score differentiated two groups (0-1.5 points; ≥ 2.5 points) with distinct prognosis and a higher ART score was associated with major adverse events after the second TACE^[60]. The same authors demonstrated that the sequential assessment of the ART-score identifies patients with dismal prognosis prior to each TACE session^[61].

TA(C)E PRE-TRANSPLANTATION FOR PATIENTS ON THE WAITING LIST

Locoregional therapies are increasingly used for patients

on the transplant waiting list despite the lack of conclusive data, in order to prevent further growth of the tumor and thus ensure that the patient remains eligible for transplantation until an organ becomes available^[62]. The recent EASL and European Organization for Research and Treatment of Cancer guidelines for HCC recommend neo-adjuvant treatment pre-transplant if the waiting list time is more than 6 mo to prevent dropouts due to tumour progression^[5]. This was partly based on a Markov model analysis that did not evaluate waiting list times of less than six month^[63]. Percutaneous techniques, although effective, are not routinely used in the pre-transplant setting in our center because of the small, but not negligible risk, of tumour seeding^[64].

We recently published our prospectively collected data of patients with HCC treated with TAE on the liver transplant waiting list and we found that pre-transplant TAE significantly reduced post-transplant HCC recurrence in patients within the Milan criteria^[65]. We have further demonstrated that the reduced calcineurin-inhibitor exposure in the first month post-transplant is associated with reduced HCC recurrence^[66]. Characteristics of tumor response to TACE on the transplant waiting list add a dynamic assesment of tumor biology and were recently suggested as potentially useful in identifying suitable patients for transplantation based on preliminary data from 136 patients^[67]. Nevertheless, conclusive data on the effects of TA(C)E on dropout rates are lacking.

TACE COMBINED WITH ANTI-ANGIOGENIC THERAPY

Sorafenib, a multikinase inhibitor with anti-angiogenic activity, became in 2008 the first systemic therapy that showed a survival benefit in patients with HCC^[68]. Theoretically, sorafenib could inhibit the growth factors such as VEGF that are synthesized in the tumor tissue in response to the TACE-induced hypoxia and therefore sorafenib may be beneficial as an adjuvant treatment with TA(C)E^[69]. Conclusive data from phase III trials to support this hypothesis are currently lacking. In a single arm, phase II study, sorafenib was administered 3 d after TACE for a total period of up to 24 wk, and resulted in a 6-mo progression free survival of 52% with an acceptable safety profile^[70]. Similarly, an interim analysis of the START trial, which is a phase II single arm trial, reported an overall response rate of 52% and no unexpected side-effects^[71]. Several trials on combinations of TACE with sorafenib but also other agents such as brivanib, sunitinib and thalidomide are registered and currently recruiting^[9,70]; a full listing is beyond the scope of these article. Until the results of such RCTs become available, combinations of TACE with targeted therapies should be performed in the context of clinical trials.

CONCLUSIONS-FUTURE DIRECTIONS

TAE and TACE should be regarded as equally effective in the management of patients with HCC; their main

indication is in patients with intermediate HCC. However they are increasingly used for patients on the liver transplant waiting list in order to prevent further tumor progression. The absence of chemotherapy may make TAE better tolerated particularly in patients with borderline liver function. Despite its use for over two decades, TA(C)E remains an unstandardized procedure, with variations in the size and type of embolizing particles, choice and dose of chemotherapeutic agent, and interval between procedures. Smaller embolising particles may result in more selective embolisation with less damage to surrounding non tumorous tissue. DEB-TACE, is not more effective than conventional TACE, but might contribute towards the standardization of the technique. The results of various combination trials of TA(C)E with sorafenib and other targeted therapies are eagerly awaited and might further improve survival in this patient group.

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WJG 20th Anniversary Special Issues (1): Hepatocellular carcinoma

Oxidative damage in the progression of chronic liver disease to hepatocellular carcinoma: An intricate pathway

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and neoplasia have been widely investigated, and it has been well established that inflammatory cells recruited at these sites with ongoing inflammatory activity release chemokines that enhance the production of reactive oxygen species. The latter, in turn, probably have a major pathogenic role in the continuum starting from hepatitis followed by chronic inflammation, and ultimately leading to cancer. The relationship amongst chronic liver injury, free radical production, and development of HCC is explored in the present review, particularly in the light of the complex network that involves oxidative DNA damage, cytokine synthesis, telomere dysfunction, and microRNA regulation.

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Key words: Reactive oxygen species; Viral hepatitis; Hepatocellular carcinoma; Telomere dysfunction; Cytokines; mitochondria; Antioxidant mechanisms; MicroRNA and circulating free DNA

Abstract

The histo-pathologic and molecular mechanisms leading to initiation and progression of hepatocellular carcinoma (HCC) are still ill-defined; however, there is increasing evidence that the gradual accumulation of mutations, genetic and epigenetic changes which occur in preneoplastic hepatocytes results in the development of dysplastic foci, nodules, and finally, overt HCC. As well as many other neoplasias, liver cancer is considered an "inflammatory cancer", arising from a context of inflammation, and characterized by inflammation-related mechanisms that favor tumor cell survival, proliferation, and invasion. Molecular mechanisms that link inflammation

Core tip: In this review, the relationship amongst chronic liver injury, free radical production, and development of hepatocellular carcinoma is explored. The review confirms the existence, in the intricate pathway involved in the progression of virus-related liver injury to cirrhosis and cancer, of a link between oxidative genomic and mitochondrial damage and telomere dysfunction. This link develops in the context of inflammatory response and induces a derangement of mechanisms controlling liver proliferation. In this scenario, mitochondria are emerging as a possible target for new treatments aimed at counteracting oxidative damage and disease progression to cancer, given their relevant role in inflammation and carcinogenesis.

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EPIDEMIOLOGY

Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver, the fifth most common cancer in men worldwide, and the seventh most frequent in women, with over 600000-650000 new cases diagnosed annually. Hepatitis B virus (HBV) and hepatitis C virus (HCV)-related chronic liver diseases are responsible for the majority of HCC cases, making this neoplasia potentially preventable.

The distribution of HCC presents a sharp variability according to the geographic area involved. In general terms, there are three major areas with different incidence rates: Eastern Asia and sub-Saharan Africa, with incidence rates that peak at 100/100000 individuals, Mediterranean countries such as Italy, Spain, and Greece with intermediate rates (10-20 per 100000 individuals), and North and South America, with a relatively low incidence (< 5 per 100000). In regions at high incidence, the most common cause is HBV transmitted at birth, while in North America and Europe the most common etiology is HCV, with infection acquired much later in life.

Male gender is a risk factor for HCC, that is in fact more common in men than in women, partly dependent upon factors other than viral infection, such as the hormonal and immunological status, as well as alcohol consumption. The major established risk factor is cirrhosis, insofar as 80%-90% of cases of HCC occur in the setting of cirrhosis^[1].

ETIOLOGY

A variety of factors have been associated with the development of HCC, including hepatitis viruses, heavy alcohol intake, nonalcoholic fatty liver disease, aflatoxin B1 exposure, obesity, diabetes, dietary habits, and iron accumulation^[2]. The presence and importance of these factors vary according to the geographical region, thereby influencing preventive measures that may be enacted, prognosis of patients, and treatment recommendations^[3]. The incidence of HCC is rising rapidly in some, but not in all, Western countries, and is declining in Europe^[4] in relation to the distribution of risk factors (hepatitis C infection, alcoholism, and obesity)^[5]. Recent data indeed demonstrate that HCV is now declining as a risk factor for HCC^[6]. It is well established that both HBV and HCV cause malignant transformation and lead to HCC development through direct and indirect mechanisms. The former involve viral proteins and specific mutations induced by the integration of the virus into the host genome, which is particularly

true for HBV infection. Indirect mechanisms, in contrast, imply the induction of chronic inflammation by the immune response elicited. The time course of virus-related liver damage and the development of HCC is different based on the virus considered: progression of the disease to HCC in HCV-infected patients requires nearly 10 years from the establishment of cirrhosis, and approximately 30 years from the initial exposure to the virus. On the other hand, the course of HBV infection is less predictable, and HCC may thus precede the occurrence of cirrhosis^[1,7].

INFLAMMATION AND OXIDATIVE STRESS

In the last twenty years, the main focus of our research group has been to investigate the events related to oxidative stress occurring in the natural history of viral hepatic disease, with a particular interest in the pathways of HCC development resulting from HBV- and HCV-related damage. The relationship between chronic inflammation and the risk of cancer development is well known. Virchow established more than a century ago the link between cancer and inflammation, and a bulk of evidence from published and ongoing studies contributes to elucidating the molecular mechanisms at the basis of this process. Inflammation involves macrophages, mast cells, dendritic and natural killer cells that are recruited within damaged tissues, and which release chemical mediators such as cytokines, chemokines, and reactive oxygen species (ROS)^[8]. The most important ROS include free oxygen radicals like superoxide ($O_2^{\bullet-}$), hydroxyl radical (OH^{\bullet}), nitric oxide (NO^{\bullet}) radicals, as well as non-radical ROS like hydrogen peroxide (H_2O_2), organic hydroperoxides, and hypochloride. A physiologic amount of ROS plays a key role in several cellular processes including proliferation, apoptosis, cell cycle arrest, and cell senescence. Different anti-oxidant mechanisms regulate ROS production, but when generated ROS exceed the basal amount and the cellular defenses, they can damage cell macromolecules including proteins, lipids, and nucleic acids (DNA and RNA). Under these conditions, ROS might play a major role in the genesis of different chronic diseases and, particularly, in the initiation/promotion phase of carcinogenesis. In particular, ROS may stimulate the growth of malignant cells or increase the activity of carcinogenic xenobiotics by facilitating their activation to reactive compounds. Among the many ROS produced during the inflammatory process, the most damaging is OH^{\bullet} , which is responsible for a number of lesions. When DNA is attacked by ROS, stable covalent bonds are produced, leading to the formation of base modifications, including the formation of thymine and thymidine glycol, 5-hydroxymethyluracil, and 8-hydroxydeoxyguanosine (8-OHdG). 8-OHdG, the main ROS-induced DNA adduct, generates a point mutation in the DNA daughter strands. In fact, studies using DNA templates containing 8-OHdG indicate that the adduct accumulates and causes mispairing, thus suggesting that

this lesion is mutagenic and therefore potentially carcinogenic. Thus, 8-OHdG is used as a reliable marker not only of oxidative stress^[9] but also of cancer risk.

Results in the literature suggest that both oxidative and nitrative DNA damage occur at the sites of carcinogenesis, regardless of the etiology of the disease. Inducible nitric oxide synthase (iNOS) produces NO through a reaction that converts arginine and oxygen into citrulline. NO is a major mediator of chronic inflammation and participates in the regulation of cell proliferation, survival, migration, angiogenesis, DNA repair, and drug resistance. Therefore, excessive amounts of reactive nitrogen species produced *via* iNOS in chronic inflammation may play an important role in tumorigenesis^[10] and it has been suggested that selective targeting of iNOS may prove a useful therapeutic or chemopreventive measure^[11,12].

An increased production of ROS has been documented in virus-related disease, with a strong link between HCV core protein or HBV X protein on one hand, and oxidative “burst” on the other, particularly in the first phases of carcinogenesis^[13]. Our own findings have suggested a progressive build-up of genomic oxidative damage which takes place not only in patients with chronic hepatitis and cirrhosis, but also in anti-HCV positive patients with persistently normal ALT levels^[14]. Our data on 8-OHdG in HCV-related liver damage have been confirmed by others, such as Shimoda *et al.*^[15], who also reported increased levels of 8-OHdG in DNA extracted from liver tissues and leukocytes of individuals with chronic HCV-related liver disease^[16,17]. New results on HBV-related liver damage have shown a delayed accumulation of oxidative DNA damage with respect to HCV patients, which significant increases only in the later stages of the disease, in association with a significant accumulation of fibrosis, and this is particularly true in livers of patients in whom cancer develops (unpublished data).

OXIDATIVE STRESS AND DNA REPAIR ENZYME

Like any other DNA damage, 8-OHdG undergoes a specific repair process. A human DNA glycosylase/AP lyase encoded by the *OGG1* gene removes 8-OHdG directly from DNA and suppresses its mutagenic effect. Among the multiple *OGG1* isoforms, *OGG1*-type 1a is expressed mainly in human cells and repairs chromosomal DNA. The human *OGG1* gene maps to chromosome 3p26.2 and allelic deletions of this region frequently occur in a variety of human cancers. Inactivation of the *OGG1* gene in yeast and mice leads to high rates of spontaneous mutation in the cells. The gene is also somatically mutated in certain cancer cells, and is highly polymorphic among human populations. The repair activity of mutated and polymorphic *OGG1* protein is lower than that of the wild-type *OGG1* protein, and may consequently be involved in human carcinogenesis, though full agreement on the point has not been reached^[18,19].

We evaluated *OGG1* gene polymorphism in HBV- and HCV-related hepatitis tissue samples. No significant difference was found between 8-OHdG levels evaluated in wild type compared with heterozygous patients, nor in HCV or HBV patients with HCC, nor in chronic hepatitis and cirrhosis, therefore downsizing the relevance of *OGG1* gene polymorphism in liver disease (unpublished data).

OXIDATIVE STRESS AND MITOCHONDRIA

In normal healthy cells, mitochondria are involved in several fundamental cellular processes, including cell proliferation, apoptosis, and intracellular calcium homeostasis. Mitochondrial dysfunction can affect a range of important cellular functions and can result in a variety of diseases^[20]. Emerging evidence strongly supports a key role of mitochondria in carcinogenesis. In conditions of oxidative stress, the transcriptional and replication machinery of mitochondria is up-regulated, thus resulting in increased mitochondrial biogenesis *via* replication of the mitochondrial genome (mtDNA). In these damaged mitochondria, the electron transport chain may be blocked, resulting in an accumulation of ROS. As mitochondrial DNA is located close to the source of ROS production, DNA itself can become damaged, resulting in accumulation of deletions and mutations^[21]. Increased levels of ROS also alter mitochondrial metabolism, increasing mitochondrial membrane permeability and leading to the release of pro-apoptotic factors into the cytosol. Research is now more clearly defining the molecular mechanisms and the signaling pathways involved in the process of “mitochondrial malignancy”. These pathways will become clearer as the respective roles of ROS and of cancer-related proteins such as RAS, p53 and c-myc in regulating mitochondria will be clarified^[22].

OXIDATIVE STRESS, ANTI-OXIDANT DEFENSES AND DRUGS

Cells have developed defense mechanisms to counteract the negative effects of oxidative stress, among which redox active glutathione, thioredoxin, antioxidant enzymes (superoxide dismutase, catalase and glutathione peroxidases)^[23]. These aspects are particularly relevant in view of the possible application of anti-oxidants in cancer prevention. Conventional anti-oxidants show little efficacy against oxidative stress *in vivo*, probably because they are not taken up by mitochondria. Mitochondria-targeted anti-oxidants such as Vitamin K3 or Vitamin E may be used to decrease mitochondrial oxidative damage, and drugs such as mitoquinone have been developed in this regard. This novel class of compounds combines a potent anti-oxidant, such as ubiquinone, with a lipophilic cation that causes the anti-oxidant to accumulate several hundred-fold within mitochondria. Several *in vitro* and animal studies have shown that this selective accumulation renders mitoquinone more

protective against ROS and cell death than untargeted anti-oxidants. The administration of the above compounds may constitute a novel approach to decreasing liver damage in chronic HCV infection. Potentially, anti-oxidants could be used in association with the standard interferon/ribavirin treatment in patients with chronic HCV-related hepatitis^[24,25]. Yet very few studies have been published so far on the effects of anti-oxidants in patients with chronic HCV infection. Alpha tocopherol and fermented papaya both improved redox status in HCV-related cirrhosis, but only in the subgroup of vitamin E-deficient patients was normalization of transaminase levels obtained^[26]. In another study, a tomato-based food supplement increased serum lycopene and carotenoids, and decreased serum hydroperoxides in patients with HCV infection, again with no effect on transaminases^[27]. In yet another study, Viusid[®], a nutritional supplement containing ascorbic acid, zinc and glycyrrhizin, of recognized anti-oxidant properties, achieved reduction of serum oxidative stress markers in patients with chronic HCV-related hepatitis; no effect on the evolution of liver damage was reported, however^[28]. Finally, in another study, a combination of different anti-oxidants at the “appropriate” dose for twenty weeks achieved normalization of liver enzymes levels in 44% of treated patients, decreasing viral load in 25%^[29]. Studies recently published by our group have demonstrated that coffee consumption induces a significant reduction of oxidative DNA damage, thus confirming that the protection exerted by coffee with respect to HCC is mediated by a reduced accumulation of oxidized bases, and consequently, of DNA mutations. The relevance of oxidative stress in carcinogenesis^[30] and, particularly in patients with HCV infection^[17,31], is well known, and the association between coffee consumption and lower oxidative DNA damage has been recently observed also by Mišák *et al.*^[32] in healthy volunteers. The protective effect of coffee with respect to HCC may be due to the numerous anti-oxidant compounds contained in this beverage, among which are polyphenols, or to the induction of antioxidant enzymes. This effect is exerted even at very small doses or even when coffee is consumed for a relatively short period, as in our above quoted study. Confirming what above, long-term glycyrrhizin administration reportedly reduces HCC incidence in interferon-resistant patients with chronic HCV infection^[33], as demonstrated in a cohort study, which although not randomized, included a large series of patients and produced results which are sound enough to be considered as relevant.

OXIDATIVE STRESS, CYTOKINES AND APOPTOSIS

As has been amply discussed herein, liver injury is associated with chronic inflammation; in a local inflammatory milieu, several cell types normally residing in the liver (sinusoidal, endothelial and Kupffer cells) produce immune mediators as well as cytokines and chemokines, whose receptors are located on the cell surface of hepatocytes.

These cells also express and release interleukin (IL)-6,

an important pro-inflammatory cytokine involved in tumor cell proliferation by its role in inhibiting apoptosis through the activation of signal transducer and activator of transcription 3^[34]. Tumor necrosis factor- α (TNF- α), a pro-inflammatory immune mediator produced by Kupffer cells and other immune cells in response to tissue injury, triggers the production of other cytokines that, in turn, recruit inflammatory cells, promoting fibrogenesis and further activating the oxidative burst^[35]. Amongst the myriad effects of TNF- α is, importantly, the activation of intracellular apoptotic and/or anti-apoptotic pathways. The role of TNF- α expression in HCC, however, remains controversial, with reports of expression varying from high^[36] to low^[37,38]. The question that ensues is henceforth to establish how persistent oxidative stress fits into this scenario. TNF- α also has an important role in oxidative stress induction, causing DNA damage through the formation of 8-OHdG in primary murine hepatocytes^[39]. The overproduction of oxidative species linked to the over-expression of inflammatory cytokines may be responsible for inhibiting the apoptotic process, probably by activating the nuclear factor (NF)- κ B-dependent pathway^[40]. Oxidative stress is also related to the expression of proto-oncogenes, such as c-myc, which is significantly more expressed in cirrhotic than in non-cirrhotic tissues in our experience as well. This means that the progression of tissue damage from hepatitis to cirrhosis, and the related cell growth changes, may be to some degree mediated by c-myc^[41]. For instance, recent studies have demonstrated that TGF- α /c-myc double transgenic mice exhibit enhanced cell proliferation and accumulate extensive oxidative DNA damage, a phenomenon that may account for an accelerated progression to cancer^[42]. Recently, we sought the possible correlations between oxidative DNA damage and levels of pro-inflammatory cytokines, TGF- α and c-myc in chronic HCV-related liver damage, and a clear correlation between 8-OHdG levels and c-myc expression has been detected, confirming the relevance of oxidative DNA damage in liver carcinogenesis^[17]. As far as IL-1 β is concerned, recent reports have shown that higher levels of this cytokine are present in HCV-related liver disease with respect to other forms of liver damage^[43] and that its polymorphisms are correlated with the risk of progression to HCC. IL-1 β has been reported to trigger the inflammatory response cascade and to directly cause growth arrest and TNF- α expression induction^[44,45]. This last effect was not confirmed in our experience, however, since no correlation emerged between IL-1 β and TNF- α levels in liver tissues of patients with HCV-related liver damage^[46]. On the other hand, IL-1 β expression was higher in the later stages of HCV-related liver disease, as already demonstrated by Gramantieri *et al.*^[47].

OXIDATIVE STRESS AND EPITHELIAL-MESENCHYMAL TRANSITION

Epithelial-mesenchymal transition (EMT) is a biologic process by which epithelial cells undergo changes that induce the development of a mesenchymal phenotype,

increasing the production of extracellular matrix proteins and the resistance to apoptotic cell death. These changes are considered as a driving force also in tumor progression since they enhance the cell migratory ability and invasiveness.

Several studies demonstrated the existence of a strong correlation between ROS production and EMT, a link that involves NF- κ B activation in collaboration with hypoxia-related production of hypoxia-inducible factor-1 and cyclooxygenase-2^[48]. HCV and HBV infections are involved in prompting EMT in the development of HCC, a process in which the progression of malignant hepatocytes depends in part on the signaling of transforming growth factor- β (TGF- β), produced by stromal cells (fibroblasts, macrophages *etc.*)^[23]. TGF- β activation triggers an increase in intracellular ROS production in association with the phosphorylation of Smad2, p38 and ERK1/2^[49].

Many other factors correlated with ROS production are involved in EMT, such as mitogen-activated protein kinase activation and angiotensin II, E-cadherin and α -SMA up-regulation, thus supporting a redox-mediated regulation of EMT^[23].

OXIDATIVE STRESS AND TELOMERE DYSFUNCTION

Cells that are not able to start the process of apoptosis, in particular after DNA damage, can be more susceptible to genetic alterations and to the acquisition of immortality through the modulation of telomerase activity. Telomerase, a RNA-dependent DNA polymerase, is a complex ribonucleoprotein including two components, a catalytic subunit (TERT) and a RNA component complementary to telomeric sequences (TR). After retrotranscription of its own RNA, telomerase adds telomeric sequences to chromosomal terminal portions, thus maintaining the length of telomeres, whose main function is to stabilize the chromosomal structure, endowing this molecule with a very important role in cell proliferation, senescence, immortalization and carcinogenesis^[50]. Nevertheless, telomeres shorten at each replication cycle, and lose their function after reaching a critical length (telomeric crisis). Telomere shortening may result in end-to-end fusions during the cell cycle and, consequently, somatic cells stop proliferation and enter senescence phase and apoptosis^[51]. In neoplastic cells, telomeric shortening, senescence, and apoptosis are avoided by an increased telomerase function. Telomere shortening and chromosomal instability occur in the first phases of carcinogenesis, while tumor progression is linked to a telomeric preservation induced by a restarting of telomerase activity. In fact, only cells that maintain telomere length, with unlimited cell divisions and chromosomal instability, possess a higher potential of neoplastic transformation and progression to cancer. The telomeres, rich in guanines, are highly sensitive to ROS attack, in particular by hydroxyl radical. ROS interaction with telomeric sequences creates DNA adducts as 8-OHdG. Thus, oxidative stress can accelerate

telomeric shortening also because, unlike in most genomic DNA, the repair mechanisms of telomeric DNA are less efficient and telomeres more easily accumulate oxidative damage. Consequently, measuring telomere length may constitute an optimal biomarker of chronic oxidative stress^[52]. Only a few studies have specifically examined the relationship between telomerase activity, telomere length and the extent of oxidative stress and consequent oxidative DNA damage in hepatocarcinogenesis. Our group has demonstrated that oxidative DNA damage interferes with telomere function, thus playing a key role in hepatic carcinogenesis (unpublished data). In fact, we confirmed the shorter telomere length in both HCV- and HBV-related HCC, possibly due to the accumulation of genetic alterations and 8-OHdG during disease progression. Alterations of the promoter may be one of the factors that control the transcriptional activity of TERT. The hypermethylation of CpG islands acts as an alternative, complementary pathway to gene mutation, and is an important mechanism involved in carcinogenesis. Both HBV and HCV induce epigenetic changes in specific genes involved in DNA repair, cell cycle control, and apoptosis signaling (RASSF1A, GSTP1, CHRNA3, and DOK1) in HCC as compared to cirrhotic or normal liver tissues^[53,54]. Our data confirm changes in the methylation levels of the TERT promoter gene in the terminal stages of the disease in both HBV- and HCV-related hepatitis. Further studies are needed to better understand the mechanisms regarding genetic and epigenetic changes induced by HBV and HCV in hepatocytes. The majority of the biological properties ascribed to TERT have been limited to its effects in the nuclear genome. Several data have been published, however, demonstrating that TERT is also targeted towards mitochondria, resulting in telomerase activity in this organelle. TERT can shuttle from the nucleus to mitochondria upon oxidative challenge. Nevertheless, the exact role of mitochondrial TERT remains controversial, with some authors sustaining that it exacerbates oxidative injury while others reporting a protective effect. Recent data in literature report that conditions of chronic oxidative stress may lead to the migration of TERT subunit of telomerase to the cytosol, following the phosphorylation of tyrosine 707 by the Src kinase, thus reducing nuclear activity of the enzyme^[55-58]. Our group has also observed TERT subunit translocation from nucleus to mitochondria in HCC tissue samples under oxidative stress. What the function of TERT is in mitochondria remains a matter of debate, but one of the most relevant hypothesis is that it may play a role in modulating apoptosis. In fact, Haendeler *et al.*^[59] demonstrated that TERT interacts with mtDNA, improving the electron transport chain activity and protecting cells from ROS-induced oxidative damage. This mechanism would reduce the permeability of the mitochondrial membrane and would avoid the leakage of pro-apoptotic factors. The reduction of apoptotic signals in a context of chronic oxidative stress, genomic instability, and increased cell proliferation might be very crucial in terms of neoplastic transformation.

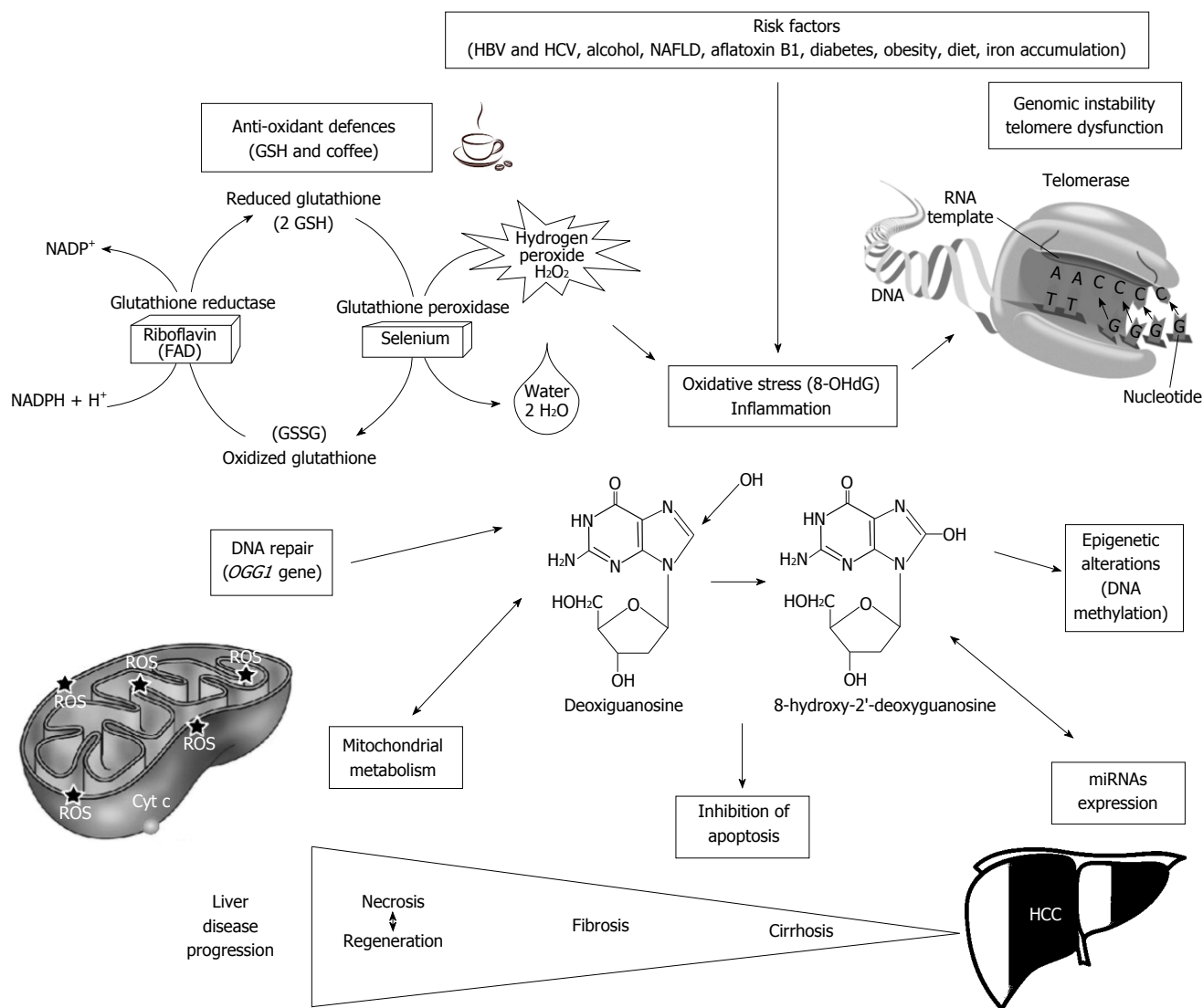


Figure 1 Intricate pathway of molecular mechanisms involved in the progression of virus-related liver injury to cirrhosis and hepatocellular carcinoma. HBV: Hepatitis B virus; HCV: Hepatitis C virus; NAFLD: Nonalcoholic fatty liver disease; HCC: Hepatocellular carcinoma.

OXIDATIVE STRESS AND MICRORNAS

Recently, the important role of microRNAs (miRNAs) in the different stages of chronic liver diseases in the development of HCC has been recognized^[60]. MiRNAs are small (21-23 nucleotides long) noncoding RNA family involved in post-transcriptional gene regulation of their target genes. In fact, miRNAs induce translational repression *via* their binding to partially complementary sequences or mRNA degradation through their binding to perfectly complementary sequences in the 3'UTR of mRNAs^[61]. Each mature miRNA potentially controls many gene targets, and each mRNA is regulated by multiple miRNAs. To date, more than 17000 distinct mature miRNA sequences have been identified from over 140 species^[62]. MiRNAs are now clearly identified as key mediators of the immune system development and function, in particular for activation in response to infection during both the innate and the adaptive immune responses. At the same time, miRNAs dysregulation is a central event in

the development of a number of cancers, as it is involved in inflammation and oncogenesis. Several miRNAs, whose expression is modulated in HCC, have been identified, again also during oxidative damage (Table 1). Our research group has recently published a significant positive correlation between miRNA-92 expression, which is already linked to hepadnavirus-associated carcinogenesis, c-myc and 8-OHdG levels in HCC tissues. Besides, our data demonstrate that miRNA-199a, miRNA-199b, miRNA-195 and miRNA-122a are strongly down-regulated in the majority (55%-70%) of HCCs, while miRNA-92 and miRNA-145 show a less marked down-regulation. In contrast, miRNA-222 is up-regulated in HCC^[72].

OXIDATIVE STRESS AND CIRCULATING FREE DNA

As previously exposed, oxidative stress is known to cause DNA damage, and cells with the greatest DNA injury die

Table 1 MicroRNAs involved in oxidative stress in different liver diseases

miRNA	Involvement in oxidative stress liver diseases	Ref.
miR-214	Alcohol induced liver disease	[63]
miR-199a-5p	Cholestatic disease	[64]
miR-122	Hepatitis C virus/NASH	[65,66]
let-7	HCC	[67]
miR-125b	Liver inflammation	[68]
miR-199a-3p	Mitochondrial dysfunction	[69]
miR-34a/miR-93	Liver aging (rat model)	[70]
miR-196	Hepatitis C virus	[71]
miR-92	HCC	[72]

HCC: Hepatocellular carcinoma; NASH: Non-alcoholic steatohepatitis.

either by necrosis or apoptosis. Oxidized DNA released from dying cells is likely the most prominent contributor to circulating cell-free DNA (cfDNA), which is a double or single stranded extracellular DNA, released by tumor apoptotic or necrotic cells and circulating in blood as a complex with histonic proteins^[73]. Low levels of cfDNA can be detected as well in healthy individuals, but higher levels characterize patients with a number of diseases including cancer^[74]. Our recent study has investigated the time course of cfDNA levels in patients in different stages of liver damage, from chronic hepatitis to cirrhosis and cancer. Data have shown that cfDNA is detectable in a small share of these last patients, but in a substantially similar percentage of CIRR and HCC patients. In HCC however, cfDNA levels are, on average, two times higher than in CIRR and, by choosing the right cut-off with ROC curves, the sensitivity in diagnosis is notably high. In our experience therefore, the role of the cfDNA quantitative analysis as a valuable diagnostic test is debatable, but cfDNA levels allow for patients discrimination with more advanced stages of disease, demonstrating a prognostic relevance in patients with HCC (unpublished data).

In conclusion, we confirm the existence of a link between oxidative genomic and mitochondrial damage and telomere dysfunction in the intricate pathway (Figure 1) involved in the progression of virus-related liver injury to cirrhosis and HCC. This link develops in the context of the inflammatory response and leads to a derangement of the basic mechanisms controlling liver proliferation. Unfortunately, despite the evidence of a clear role for oxidative burst as an inducer of liver damage in patients with viral hepatitis, nor antioxidant drugs nor cocktails of vitamins or different compounds have been demonstrated to actively interfering with the development of damage. In this scenario, mitochondria are emerging as a possible target for new treatments aimed at counteracting oxidative damage and disease progression as well as cancer development, given the relevant role that these organelles play in inflammation and carcinogenesis.

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WJG 20th Anniversary Special Issues (1): Hepatocellular carcinoma

Systemic therapy of hepatocellular carcinoma: Current status and future perspectives

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Abstract

The management of hepatocellular carcinoma (HCC) has substantially changed in the past few decades, the introduction of novel therapies (such as sorafenib) have improved patient survival. Nevertheless, HCC remains the third most common cause of cancer-related deaths worldwide. Decision-making largely relies on evidence-based criteria, as showed in the US and European clinical practice guidelines, which endorse five therapeutic recommendations: resection; transplantation; radiofrequency ablation; chemoembolization; and sorafenib. Many molecularly targeted agents that inhibit angiogenesis, epidermal growth factor receptor, and mammalian target of rapamycin are at different stages of clinical development in advanced HCC. Future research should continue to unravel the mechanism of hepatocarcinogenesis and to identify key relevant molecular targets for therapeutic intervention. Identification and validation of potential surrogate and predictive biomarkers hold promise to individualize patient's treatment to maximize clinical benefit and minimize the toxicity and cost of targeted agents.

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Key words: Hepatocellular Carcinoma; Molecular agents; Targeted therapy; Sorafenib

Core tip: Many molecularly targeted agents that inhibit angiogenesis, epidermal growth factor receptor, and mammalian target of rapamycin are at different stages of clinical development in advanced hepatocellular carcinoma. Future research should continue to unravel the mechanism of hepatocarcinogenesis and to identify key relevant molecular targets for therapeutic intervention.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a relevant health problem, being the sixth most common cancer worldwide in terms of incidence with 626000 new cases per year, accounting for 5.7% of all new cancer cases^[1]. Due to the poor prognosis of the disease, the number of deaths per year is almost the same as new cases (598000), making HCC the third most common cause of cancer-related death^[1].

Prognosis and feasibility of treatments for HCC patients largely depend not only on tumor characteristics, but also on the severity of the underlying chronic liver disease that affects the majority of cases^[2,3]. Outcome is significantly worse for those patients who can be treated only with palliative loco-regional treatments, such as transcatheter arterial chemo-embolization, or who are affected by advanced disease. Unfortunately, curative strategies are currently limited to a minority of patients, those who present at diagnosis with small nodules, dis-

ease confined to the liver, good performance status and well preserved liver function. The proportion of patients presenting with these characteristics is currently no more than about 30%-40%^[4]. In the experience of the Cancer of the Liver Italian Program group, in a series of 650 patients diagnosed in the years 1994-1999, 59% of patients at diagnosis were not treatable by surgery or percutaneous ablation^[5]. However, the proportion of small, early tumors is expected to significantly increase in the next years, together with the diffusion of surveillance procedures of high-risk patients, allowing tumor diagnosis at an earlier stage^[4].

Although HCC can be considered a common cancer, evidence about best treatment options is currently based on a disappointingly limited number of randomized controlled trials, compared to many other solid tumors.

SYSTEMIC THERAPY

Unfortunately, a relevant proportion of patients successfully treated with surgical resection or local ablation therapies will experience tumor relapse. Several clinical trials have been conducted to test the efficacy of adjuvant treatments following surgical resection or complete necrosis obtained with ablation.

Several trials testing the role of interferon showed beneficial effect^[5]. Most trials were characterized by a small sample size, and interferon, which is associated with significant side effects, cannot currently be considered a standard adjuvant strategy. The recent availability of target-based agents will offer a number of new approaches to test in this setting. From this point of view, the multitarget inhibitor sorafenib appears particularly promising, after the good results obtained in the treatment of advanced disease. A randomized phase III study, the STORM trial, comparing sorafenib to placebo as adjuvant treatment for patients who have received surgical resection or local ablation is close to enrol and is currently ongoing.

Occurrence of extrahepatic disease at relapse (*e.g.*, lymph-node involvement or distant metastases) is obviously associated with a significantly worse prognosis. However, recurrence consists frequently of intrahepatic disease only, in this case it can be divided in local recurrence and distant intrahepatic recurrence. Following ablation therapy (like percutaneous ethanol injection or radiofrequency ablation), tumor can recur in the site already treated. In this case, local recurrence can be attributed to incomplete tumor necrosis obtained with the previous ablative treatment. On the contrary, intrahepatic distant recurrent disease following surgical resection or after local ablation has in principle a double etiology: it can represent intrahepatic metastasis, related to previously treated tumor, or can be expression of multicentric disease, unrelated to the primary nodule but arising in the same underlying liver disease. To date, treatment of intrahepatic distant recurrences after curative treatment of primary tumor is commonly based, similarly to primary tumor, on patient's characteristics, on liver function

and on number and location of nodules. The difference between intrahepatic metastasis and multicentric occurrence, due to the objective difficulty of a correct differential diagnosis, is not accounted for in existing treatment guidelines. However, at least from a theoretical point of view, the ability to differentiate intrahepatic metastases from multicentric nodules could have relevant implications on treatment strategy. A newly diagnosed second nodule, if expression of multicentric disease, can be effectively treated with a potentially curative loco-regional approach, similarly to primary tumor. These treatments will probably be less effective if the nodule is expression of metastatic disease. In the latter case, disease should be considered at a more advanced stage, and could probably benefit, in addition or in alternative to loco-regional treatment, from a systemic treatment for advanced disease.

Until recently, systemic therapy of advanced HCC provided marginal benefit if any^[6]. Systemic chemotherapy for HCC has been associated with low response rates and no survival benefit, partly because HCC is a chemotherapy-resistant tumor^[7] - due to the expression of the multi-drug resistance gene *MDR-1*^[7,8] - and partly due to the underlying liver cirrhosis in most patients, which prevents the administration of full dosage of many drugs. In addition, the majority of controlled clinical trials of systemic therapy in this patient population are flawed by inappropriate endpoints and controls, as well as by inadequate sample size.

In the following paragraphs, we briefly describe the modest results obtained in the past with hormonal therapy, with chemotherapy and with biological and biochemical treatment. Subsequently, we describe the promising results recently obtained with molecularly targeted agents.

HORMONAL THERAPY

Hormonal therapy of HCC has been investigated based on the finding that various hormone receptors are present in HCC, with a possible association between estrogen and tumor^[9].

The finding that various hormone receptors are present in HCC has led many investigators to examine the role of hormone manipulation in this disease. Several lines of evidence have suggested an association between estrogen and HCC. Estrogen receptors are expressed in normal human liver, in chronic hepatitis, in benign hepatic tumour tissues, and rarely in HCC at a low concentration. In preclinical models, estrogens are involved in stimulating hepatocyte proliferation *in vitro* and may promote liver tumour growth *in vivo*. The persistent administration of estrogens, particularly in the form of oral contraceptives, has been associated with an increased incidence of hepatic adenomas and a small increased incidence of HCC. Tamoxifen, an antiestrogenic compound, has been shown to reduce the level of estrogen receptors in the liver. Tamoxifen has been extensively studied in HCC. Six large randomized studies (four of which were double-blind trials) have failed to demonstrate improved

survival with tamoxifen in advanced HCC^[10,11]. The role of anti-androgen therapies has been investigated, as well, but they have also failed to improve survival in randomized studies in patients with advanced HCC^[12].

In a small prospective randomized Greek study, conducted in 58 patients with advanced HCC, subcutaneous octreotide (250 µg twice daily) was associated with a median survival time of 13 mo, compared with only 4 mo in the group who received no treatment^[13]. These results were really promising but later on, another placebo-controlled study randomized 70 patients with advanced HCC to receive a 2-wk course of 250 µg of short-acting octreotide twice daily, followed by a long-acting octreotide 30-mg injection once every 4 wk for six doses, or placebo^[14]. Unfortunately, there was no difference in median survival time between the two groups. However, the median survival time was less than 2 mo in both groups, indicating that the study recruited patients with a very poor prognosis, who are very unlikely to derive benefit from any medical therapy. A more recent trial randomized 120 patients with advanced HCC to long-acting octreotide or placebo, with no difference in median survival (4.7 mo with octreotide and 5.3 mo with placebo)^[15]. Barbare *et al*^[16] reported the preliminary results of a randomized placebo-controlled trial. Two hundred 72 patients with unresectable HCC were randomized to receive either octreotide (monthly *im* injection of 30 mg of long-acting octreotide) or a placebo. Again, no survival benefit was seen in the results of the interim analysis after the occurrence of 150 deaths: the median overall survival time was 6.5 mo in the octreotide arm and 7.3 mo in the placebo arm. Finally, the results of another multicenter randomized trial assessing the combination of long-acting octreotide and tamoxifen in 109 patients with HCC were recently published, again with negative results^[17].

Two studies correlated the expression of somatostatin receptors in HCC and the response to octreotide, reaching conflicting conclusions. In one study, patients with HCC expressing somatostatin receptors and randomized to receive octreotide showed a significantly improved survival compared to placebo^[18], while in another study there was no relationship between expression of somatostatin receptors by HCC and response to octreotide^[19].

In conclusion, octreotide does not seem to benefit patients with advanced HCC. Whether octreotide may have limited benefits in advanced HCC patients whose tumors express somatostatin receptors remains to be defined.

Although a large number of controlled and uncontrolled studies have been performed with most classes of chemotherapeutic agents, no single or combination chemotherapy regimen is particularly effective in HCC. The response rate tends to be low, and the response duration is short. The response criteria used in some of the earlier studies were poorly defined. Most of the earlier studies did not stratify patients on the basis of the severity of underlying cirrhosis or other factors, making comparison of study results difficult. More importantly, any survival benefit of systemic chemotherapy for HCC remains to

be determined.

CHEMOTHERAPY

Doxorubicin is perhaps the most widely used agent in HCC. Despite the initial encouraging reports from Uganda for single-agent doxorubicin, subsequent studies have failed to confirm these data. In a large study of doxorubicin in advanced HCC, no responses were noted among 109 patients^[20]. Among 475 patients who received doxorubicin in various studies, a 16% response rate was documented, with a median survival of 3-4 mo^[21]. Systemic therapies that have not demonstrated improved overall survival benefits in advanced hepatocellular carcinoma.

A variety of combination chemotherapy regimens has been studied in HCC. Although a few of them have shown improved response rates, most of these have not been studied in large randomized phase III studies. The most impressive results from phase II studies are from the chemotherapy regimen that uses the combination of cisplatin, interferon alfa, doxorubicin, and 5-fluorouracil (PIAF)^[22]. This regimen produced a partial response (PR) rate of 26%. In 9 of the 50 patients, the initially unresectable tumours became resectable after chemotherapy. In four of these patients, the resected specimens had a pathologic complete response and the alfa-fetoprotein levels fell to within the reference range. Unfortunately, this regimen was also associated with marked hematologic and gastrointestinal toxicity. Yeo *et al*^[23] subsequently examined the efficacy of this regimen in a randomized phase III study comparing PIAF with single-agent doxorubicin. A total of 188 patients with unresectable HCC were enrolled. The median survival of the doxorubicin and PIAF groups was 6.83 mo (95%CI: 4.80-9.56) and 8.67 mo (95%CI: 6.36-12.00), respectively ($P = 0.83$), which failed to reach statistical significance for the study primary end point.

The difficulty of developing effective chemotherapy in HCC may in part be due to the inherent resistance in the tumour conferred by the multidrug-resistant gene *MDR-1*. In addition, the underlying cirrhosis present in most patients may lead to portal hypertension with hypersplenism, platelet sequestration, varices and gastrointestinal bleeding, hepatic encephalopathy, hypoalbuminemia, differential drug binding and distribution, and altered pharmacokinetics, limiting the selection and adequate dosing of most cytotoxic agents.

Two new chemotherapy drugs, nolatrexed - a novel thymidylate synthase inhibitor - and T138067 - a microtubule formation inhibitor - were compared to doxorubicin in two phase III randomized studies^[24,25]. Unfortunately, neither nolatrexed nor T138067 provided survival benefit compared with doxorubicin. At the present time, there is no cytotoxic drug or regimen that can be clearly defined as a standard for treating HCC, and chemotherapy should not be considered as an option for patients with HCC.

Without doubt, the development of molecularly targeted agents opened new and exciting perspectives for systemic therapy of HCC. Many molecular alterations

Table 1 Efficacy results of molecular agents for advanced hepatocellular carcinoma

Molecular agent	Study phase	Results	Ref.
Sorafenib	III (Sharp) <i>vs</i> placebo	Median OS: 10.7 mo <i>vs</i> 7.9 mo	[31]
	III (Asian) <i>vs</i> placebo	Median OS: 6.5 mo <i>vs</i> 4.2 mo	[32]
	II	Median OS: 13.7 mo <i>vs</i> 6.5 mo	[33]
	(sorafenib + doxorubicin <i>vs</i> doxorubicin)		[33]
Bevacizumab	II	Median OS: 12.4 mo	[43]
	II	Median OS: 9.6 mo	[45]
	(Beva + gemox)	Median OS: 15.0 mo	[48]
	II		
Sunitinib	(Beva + erlotinib)		
	II	Median OS: 9.8 mo	[49]
	II	Median OS: 8.0 mo	[50]
	III	Median OS: 7.9 mo <i>vs</i> 10.2 mo	[51]
Brivanib	(Sunitinib <i>vs</i> sorafenib)		
	II	Median OS: 9.7 mo	[52]
	III		
	BRISK-PS (Briv <i>vs</i> placebo)	Median OS: 9.4 mo <i>vs</i> 8.3 mo	[53]
ABT 869 (Inifanib)	III		
	BRISK-FL (Briv <i>vs</i> sorafenib)	Median OS: 9.5 mo <i>vs</i> 9.9 mo	[54]
	II	Median OS: 9.7 mo	[55]
	I	Median TTP 4.5 mo	[56]
Pazopanib	II	Median OS: 5.8 mo	[57]
AZA2171 (Cediranib)	I - II	Median OS: 7.3 mo	[58]
Vatalanib (PTK787/ZK 222584)	II	Median OS 7.2 mo <i>vs</i> 3.8 wk	[59]
Tivantinib (ARQ 187)	(Tivant <i>vs</i> placebo)	(c-met High)	
Ramucirumab	II	PFS 4.3 mo	[60]
Everolimus	I - II	PFS 3.8 mo	[75]
Erlotinib	II	Median OS: 13 mo	[67]
Gefitinib	II	Median OS: 6.5 mo	[69]
Lapatinib	II	Median OS: 6.2 mo	[70]
Cetuximab	II	Median OS: 9.6 mo	[71]

OS: Overall survival; TTP: Time to progression; PFS: Progression-free survival.

have been identified in HCC and a lot of work has been done to identify the potential therapeutic targets.

In the following paragraphs, we describe the promising results recently obtained with molecularly targeted agents, in particular with sorafenib, that is the first drug with high-level evidence of efficacy in patients with advanced HCC and the future perspectives with new molecular agents.

MOLECULARLY TARGETED THERAPY

In the past few years, the mechanisms of hepato-carcinogenesis have been elucidated and the involvement of a number of pathways, including angiogenesis, aberrant signal transduction, and dysregulated cell cycle control have been demonstrated, leading to the evaluation of the activity and toxicity of some of the new molecular target agents^[26] (Table 1). In chronic hepatitis and liver cirrhosis the phenotypically altered hepatocytes have high epidermal growth factor receptor (EGFR) expression and non-committal epigenetic changes (increase in transforming growth factor (TGF)- β , insulin-like growth factor-2 and Raf). These phenotypically altered hepatocytes may become dysplastic and show more committed genetic changes, *e.g.*, increased telomerase activity and varied al-

lelic deletions, which may eventually lead to the evolution of HCC with additional genetic changes, such as an increase in c-myc and decrease in p16 expression^[27].

Significant progress on the treatment of advanced HCC has been made possible by sorafenib, a novel signal transduction inhibitor that blocks tumour cell proliferation by targeting the Raf/MEK/ERK signalling pathway and exerts an antiangiogenic effect by targeting the tyrosine kinases of vascular endothelial growth factor receptor (VEGFR)-2, VEGFR-3, and platelet-derived growth factor receptor (PDGFR)-beta. In preclinical models, sorafenib exhibited antitumor activity in HCC cells and xenograft models. In a phase II study of 137 patients with advanced HCC, sorafenib provided orally at 400 mg twice daily induced a PR in 2.2% of patients, a minor response in 5.8%, and stable disease lasting 4 mo in 34%^[28]. Median time to progression (TTP) was 4.2 mo, and median overall survival (OS) was 9.2 mo. The international, phase III, placebo-controlled sorafenib HCC Assessment Randomized Protocol trial evaluated 602 patients with advanced HCC who had not undergone prior systemic therapy to receive either sorafenib at 400 mg twice daily (299 patients) or placebo (303 patients)^[29]. The primary end point of the study was OS. Patients with underlying Child-Pugh A cirrhosis accounted for 95% and 98% in

the sorafenib and placebo groups, respectively. Median OS was 10.7 mo in the sorafenib group and 7.9 mo in the placebo group (HR = 0.69; $P = 0.001$). The median TTP was 5.5 mo in the sorafenib group and 2.8 mo in the placebo group ($P = 0.001$). In another Asian-Pacific randomized phase III study, sorafenib also demonstrated improved OS in patients with advanced HCC, mostly in patients with hepatitis B virus infection^[30]. OS was 6.5 mo in the sorafenib group *vs* 4.2 mo in the placebo group (HR = 0.68; $P = 0.014$). The safety profiles of sorafenib seem favorable; however, grade III diarrheal, hand-and-foot skin reaction, and fatigue were observed. The successful development of sorafenib has validated the use of molecularly targeted agents in HCC. This is the first agent ever to have shown improved survival benefits in this disease. It highlights the importance of selecting the right patient population (good performance status and preserved hepatic function) for clinical trial design. The major benefits of sorafenib are mainly manifested as disease stabilization rather than radiologic response. However, many questions remained unanswered: what is the mechanism of action mediating the clinical benefits of sorafenib? Who are at risk for developing toxicities? What is the escape and resistance mechanism of sorafenib failure? Will sorafenib benefit patients with worsening underlying cirrhosis? Will sorafenib prove to be beneficial in patients in earlier stages of disease that is, after surgical resection, high-risk transplantation, or radiofrequency ablation, as well as transarterial chemoembolization? Some of these questions are addressed in ongoing and planned clinical trials as BOOST trial.

Sorafenib-based regimens under development

Abou-Alfa *et al*^[31] reported their experience from a randomized, double-blinded, phase II study comparing Doxorubicin in combination with sorafenib *vs* doxorubicin with placebo in patients with advanced HCC. Patients had Eastern Cooperative Oncology Group performance status of 0-2, Child-Pugh A cirrhosis, and no prior systemic therapy. They received Doxorubicin at 60 mg/m² intravenously every 21 d (cycle) plus either sorafenib at 400 mg orally twice daily or placebo, for a maximum of six cycles of doxorubicin. Patients could continue with single-agent sorafenib or placebo afterward. The primary end point was TTP by independent review. Ninety-six patients were randomized in this study. Following complete accrual, an unplanned early analysis for efficacy was performed by the independent data monitoring committee, so the trial was halted. The 2 patients remaining in the placebo group at that time were offered sorafenib. Based on 51 progressions, 63 deaths, and 70 events for progression-free survival, median time to progression was 6.4 mo in the sorafenib-doxorubicin group (95%CI: 4.8-9.2), and 2.8 mo (95%CI: 1.6-5) in the doxorubicin-placebo monotherapy group ($P = 0.02$). Median overall survival was 13.7 mo (95%CI: 8.9-not reached) and 6.5 mo (95%CI: 4.5-9.9; $P = 0.006$), and progression-free survival was 6.0 mo (95%CI: 4.6-8.6) and 2.7 mo (95%CI:

1.4-2.8) in these groups, respectively ($P = 0.006$). Toxicity profiles were similar to those for the single agents.

Among patients with advanced HCC, treatment with sorafenib plus doxorubicin compared with doxorubicin monotherapy resulted in greater median time to progression, overall survival, and progression-free survival. The degree to which this improvement may represent synergism between sorafenib and doxorubicin remains to be defined. The combination of sorafenib and doxorubicin is not yet indicated for routine clinical use.

Because of the lack of consensus on the best chemotherapeutic agents/regimens in HCC and the safety concerns including cardiac toxicity for doxorubicin, other investigators are investigating the efficacy and tolerability of combining sorafenib with capecitabine and oxaliplatin or gemcitabine and cisplatin in advanced HCC. Given the complexity of hepatocarcinogenesis and heterogeneity of HCC, targeting HCC by means of a combination of sorafenib and another agent inhibiting a distinct pathway represents an appealing strategy. On the basis of this rationale, preclinical data, phase I experience, and single-agent activity and tolerability in HCC, a randomized international phase III study comparing sorafenib plus erlotinib *vs* sorafenib plus placebo as first-line treatment in advanced HCC is ongoing. The primary end point of the study is OS. Other sorafenib-based combinations, including mTOR inhibitors and insulin growth factor receptor (IGF-R) inhibitors, are at an early stage of development.

Antiangiogenic agents and TKI-inhibitors

HCCs are vascular tumours, and increased levels of vascular endothelial growth factor (VEGF) and microvessel density have been observed^[34,35]. High VEGF expression has been associated with worse survival^[36-38]. Therefore, inhibition of angiogenesis represents a potential therapeutic target in HCC, and several antiangiogenic agents have entered clinical studies in HCC.

Bevacizumab: Bevacizumab is a recombinant humanized monoclonal antibody that targets VEGF. In addition to its direct antiangiogenic effects, Bevacizumab may enhance chemotherapy administration by "normalizing" tumour vasculature and lowering the increased interstitial pressure in tumours^[39,40]. Several studies have explored the use of Bevacizumab either as a single agent or in combination with cytotoxic or molecularly targeted agents in patients with advanced HCC. Siegel *et al*^[41] reported their experience using single-agent bevacizumab in HCC in a phase II study. Two dosages of bevacizumab, 5 and 10 mg/kg administered intravenously once every 2 wk, were tested in patients with HCC with no overt extrahepatic metastases or invasion of major blood vessels. Of the 46 patients with data available for efficacy, 6 had objective responses (13%; 95%CI: 3-23), and 65% were progression free at 6 mo. Median progression-free survival (PFS) time was 6.9 mo (95%CI: 6.5-9.1), and median survival was 12.4 mo (95%CI: 9.4-19.9). Malk *et al*^[42] also reported their early experience using Bevacizumab as a single agent in HCC

in a phase II study. The combination of Bevacizumab with cytotoxic agents was also evaluated in three phase II studies. Zhu *et al.*^[43] completed a phase II study that used bevacizumab in combination with gemcitabine and oxaliplatin in advanced HCC. This regimen had moderate antitumor activity in HCC with an overall response rate of 20% in evaluable patients. An additional 27% of patients had stable disease with a median duration of 9 mo (range, 4.5-13.7 mo). The median OS was 9.6 mo and the median PFS was 5.3 mo. The combination of Bevacizumab with capecitabine and oxaliplatin or with capecitabine alone in patients with advanced HCC was also reported^[44,45]. Thomas *et al.*^[46] reported their single-center phase II experience using the combination of Bevacizumab and Erlotinib in patients with advanced HCC. Bevacizumab was provided at 10 mg/kg intravenously once every 14 d and Erlotinib at 150 mg orally daily. Of the 40 patients with efficacy data available, a 25% response rate was observed. The median PFS was 9 mo and OS was 15 mo. The above studies demonstrated early evidence of antitumor activity of Bevacizumab in HCC. Despite the overall good tolerability profiles, the risk of bleeding, hypertension, and thromboembolic events remain to be further characterized. Moreover, as a result of the nonrandomized nature, small sample size, and patient selection bias inherent in single-arm studies, the relative contributions, if any, from any chemotherapy regimens or erlotinib remain unknown and warrant further investigations.

Sunitinib: Sunitinib is an oral multikinase inhibitor that targets receptor tyrosine kinases including VEGFR-1, VEGFR-2, PDGFR- α/β , c-KIT, FLT3, and RET kinases. Zhu *et al.*^[47] performed a study in patients with advanced HCC that used sunitinib at 37.5 mg orally once daily on a standard 4-wk-on, 2-wk-off regimen (6 wk per cycle). The primary end point of the study was PFS. Of the 34 patients enrolled, one patient had a PR of 20 mo duration, and an additional 10 patients (38.5%) had stable disease of at least 12 wk duration. The median PFS was 3.9 mo and OS was 9.8 mo. In another European/Asian phase II study, sunitinib was administered at 50 mg daily for 4 wk every 6 wk to patients with unresectable HCC^[48]. The primary end point of the study was overall response rate according to Response Evaluation Criteria in Solid Tumours criteria. Of the 37 patients enrolled, one patient (2.7%) experienced PR, and 13 patients (35%) had stable disease as their best response. The median OS was 8.0 mo and PFS was 3.7 mo. Preliminary results from two other phase II studies were also presented, one that used 37.5 mg for a 4-wk-on, 2-wk-off schedule, and the other with 37.5 mg continuous daily dosing.

In terms of toxicity, the studies that used the lower dose (37.5 mg) reported acceptable safety profiles. The most common adverse events included hematologic toxicities, fatigue, and an increase in transaminase. Grade 3 or 4 adverse events occurred in no more than 20% of the patients in any category. At the higher dose of 50 mg daily, sunitinib treatment led to more pronounced grade

3-4 toxicities and a higher death rate of 10% in this patient population.

Although the lower dose at 37.5 mg seems to be more tolerable, it remains uncertain whether the continuous or intermittent schedule is better. A randomized phase III study comparing sunitinib at 37.5 mg continuous daily dosing *vs* sorafenib at 400 mg twice daily in advanced HCC was presented at ASCO meeting in 2011 and sunitinib failed its primary OS endpoint, indeed the median OS was 7.9 mo for sunitinib *vs* 10.2 mo for sorafenib^[49].

Brivanib: Brivanib alaninate is a dual inhibitor of VEGFR and fibroblast growth factor receptor (FGFR)-signaling pathways that can induce tumour growth inhibition in mouse HCC xenograft model. A phase II study was conducted to assess the efficacy and safety of brivanib in patients with unresectable locally advanced or metastatic HCC who had received either no prior systemic therapy (cohort A) or one prior regimen of angiogenesis inhibitor (cohort B)^[50]. This phase II open-label study assessed brivanib as second-line therapy in patients with advanced HCC. Brivanib was administered orally at a dose of 800 mg once daily. The primary objectives were tumor response rate, time to response, duration of response, progression-free survival, OS, disease control rate, TTP, and safety and tolerability. Forty-six patients were treated. Best responses to treatment with brivanib (N/46 patients) using modified World Health Organization criteria were partial responses for two patients (4.3%), stable disease for 19 patients (41.3%), and progressive disease for 19 patients (41.3%). The tumor response rate was 4.3%; the disease control rate was 45.7%. Median OS was 9.79 mo. Median TTP as assessed by study investigators following second-line treatment with brivanib was 2.7 mo. The most common adverse events were fatigue, decreased appetite, nausea, diarrhea, and hypertension. In conclusion Brivanib had a manageable safety profile and is one of the first agents to show promising antitumor activity in advanced HCC patients treated with prior sorafenib. Large randomized phase III Brivanib Study in Patients at Risk (BRISK) HCC program trials have been conducted to evaluate the role of brivanib in advanced HCC (BRISK-FL, BRISK-PS and BRISK-APS). The BRISK-PS trial evaluated brivanib *vs* placebo in patients who had failed or were intolerant to sorafenib therapy. This study did not meet its primary end point of improving OS, but treatment with brivanib showed improvements in the response rate^[51]. The BRISK-FL trial directly compared the clinical outcomes of brivanib *vs* sorafenib in patients with advanced HCC who received no prior systemic therapy. The median OS was 9.5 mo in the brivanib arm compared with 9.9 mo in the sorafenib arm, which was not a statistically significant difference. No significant survival differences were observed between subgroups based on geographic regions, cause of HCC or disease severity. The study did not meet its primary OS objective based upon a non-inferiority statistical design^[52].

ABT-869: ABT-869 (inifanib) is an orally active, potent, and selective inhibitor of VEGFR and PDGFR. Preliminary results from an open-label, multicenter phase II study of ABT-869 in advanced HCC were reported^[53]. ABT-869 was provided at 0.25 mg/kg daily in Child-Pugh A or once every other day in Child-Pugh B patients until disease progressed or toxicity became intolerable. The primary end point was the progression-free rate at 16 wk. Of the 44 patients enrolled, 34 had data available for analysis (28 with Child Pugh A and 6 with Child Pugh B cirrhosis). The estimated response rate was 8.7% (95%CI: 1.1-28) for the 23 patients with Child A cirrhosis. For all 34 patients, median TTP was 112 d (95%CI: 110-not estimable), median PFS was 112 d (95%CI: 61-168), and median OS was 295 d (95%CI: 182-333). The most common adverse events for all patients were hypertension (41%), fatigue (47%), diarrhea (38%), rash (35%), proteinuria (24%), vomiting (24%), cough (24%), and oedema peripheral (24%). The most common grade 3-4 adverse events were hypertension (20.6%) and fatigue (11.8%). The early evidence of efficacy and tolerable safety profiles has encouraged further development of ABT-869 in HCC.

Pazopanib: Pazopanib is an oral angiogenesis inhibitor targeting VEGFR, PDGFR, and c-Kit. Reports from a phase I study to determine the maximum tolerated dose (MTD), safety, pharmacokinetics, pharmacodynamics, and efficacy of pazopanib in patients with locally unresectable and/or advanced HCC were presented^[54]. Eligibility criteria included unresectable and/or metastatic HCC with at least one target lesion, recovery from prior systemic regimens, Eastern Cooperative Oncology Group performance status of 0 or 1, Child Pugh A, and adequate organ function. Doses of pazopanib were escalated from 200 mg once daily to 800 mg daily in a 3 + 3 design. In the 27 Asian patients enrolled, MTD was determined to be 600 mg once daily. PR was observed in two patients (7%; one at 800 mg, one at 600 mg) and stable disease of 4 mo in 11 patients (41%). Median TTP at the MTD was 137.5 d (range, 4-280 d). Changes in tumour dynamic contrast-enhanced magnetic resonance imaging parameters were seen after repeated dose pazopanib administration.

Cediranib (AZD2171): Cediranib is a potent oral pan-VEGF receptor tyrosine kinase inhibitor with activity against platelet-derived growth factor receptors and c-Kit. AZD2171 is a potent inhibitor of both KDR (IC₅₀ = 0.002 nM) and Flt-1 (IC₅₀ = 0.005 nM), and shows activity against c-kit, platelet-derived growth factor receptor beta (PDGFRβ) and Flt-4 at nanomolar concentrations. Alberts *et al.*^[55] reported their experiences of toxicity and efficacy of AZD2171 from a phase II study in patients with advanced HCC, the median OS was 5.8 mo. No patients experienced confirmed response. The median time to progression was 2.8 mo.

Vatalanib (PTK787/ZK 222584): Vatalanib is an oral

angiogenesis inhibitor targeting all known VEGFR tyrosine kinases, including VEGFR-1/Flt-1, VEGFR-2/KDR, and VEGFR-3/Flt-4, PDGFR, and the c-kit with a higher selectivity for VEGFR-2. Koch *et al.*^[56] reported the early experience of an open-label, multicenter phase I study to characterize the safety, tolerability, and pharmacokinetic profile of PTK787 administered once daily at a dose of 750-1250 mg in patients with unresectable HCC. Patients were stratified into three groups with mild, moderate, and severe hepatic dysfunction, respectively, on the basis of total bilirubin and aspartate aminotransferase/alanine aminotransferase levels. The maximal tolerated dose of PTK787 was defined as 750 mg daily. Of patients in all groups, 18 had efficacy data available. No complete response or PR was observed. Nine patients had a best response of stable disease, and nine had progressive disease. There are no studies planned to develop this agent in the treatment of HCC at this time.

Tivantinib (ARQ 187): Tivantinib is a selective, oral inhibitor of c-Met, the tyrosine kinase receptor for hepatocyte growth factor involved in tumor cell migration, invasion, proliferation and angiogenesis. Tivantinib has shown promising results in HCC in phase I studies as monotherapy and in combination with sorafenib. A phase II study was published this year, this multi-center randomized clinical trial enrolled patients with unresectable HCC, 1 failed systemic therapy, ECOG PS < 2. Child-Pugh B-C were excluded. Patients were randomized 2:1 to oral tivantinib [360 mg *bid* (A), 240 mg *bid* (B)] or placebo (P), stratifying by PS and vascular invasion. Treatment continued until disease progression (PD) or unacceptable toxicity^[57]. RECIST 1.1 response was evaluated by CT/MRI every 6 wk. Crossover to open-label T was allowed after PD. Primary endpoint was TTP in the intent-to-treat (ITT) population by central radiology review. Other endpoints included disease control rate (DCR), PFS, OS, efficacy in Met+ (Met ≥ 2+ in > 50% of tumor at immunohistochemistry) pts, safety. Major TTP, DCR and PFS benefits were obtained in Met+ patients, with preliminary OS trend favoring Tivantinib (HR = 0.47) and no detrimental effect in Met- patients. Disease control rate (95%CI) in tivantinib/placebo was 44 (31-56)/31(16-48)% for ITT and 50(28-72)/20(4-48)% in Met+ patients. Most common AEs in Tivantinib were asthenia (26.8%), NEUT (25.4%), low appetite (25.4%); most common drug-related AEs were NEUT (25.4%), anemia (15.5%). Most frequent drug-related serious AE was neutropenic sepsis (4.2%). Efficacy was similar in A/B with less frequent NEUT in B (21.1%/6.1%). Compared to Placebo, tivantinib significantly benefited second-line HCC patients, especially if Met+, with manageable safety profile at 240 mg BID. A phase III, randomised, double blind study of tivantinib in subjects with met-diagnostic high inoperable HCC treated with one prior systemic therapy is ongoing.

Ramucirumab: The monoclonal antibody ramucirumab

is a specific inhibitor of VEGFR-2. A phase II study of 42 patients with advanced HCC and primarily well-preserved liver function (75% C-P A status) showed that first-line ramucirumab monotherapy produced a disease control rate of 50% and a median PFS of 4.3 mo^[58]. This positive study prompted the phase III REACH trial in HCC, comparing ramucirumab/supportive care with placebo/supportive care for second-line treatment after sorafenib, the results of which will be available in the coming months.

EGFR inhibitors

The expression of several EGF family members, specifically EGF, TGF- α , and heparinbinding epidermal growth factor, as well as EGFR, has been described in several HCC cell lines and tissues^[59-64]. Multiple strategies to target EGFR signaling pathways have been developed, and two classes of anti-EGFR agents have established clinical activity in cancer: monoclonal antibodies that competitively inhibit extracellular endogenous ligand binding, and small molecules that inhibit the intracellular tyrosine kinase domain. Other than the modest activity with erlotinib, the rest of the EGFR inhibitors failed to show any activity as single agents in advanced HCC.

EGFR tyrosine kinase inhibitors

Two phase II clinical studies have evaluated the safety and efficacy of Erlotinib provided at 150 mg daily in patients with advanced HCC. In the study by Philip *et al.*^[65], 3 (9%) of 38 patients experienced PR, and 12 patients (32%) were free of progression of disease at 6 mo. Median OS time for this cohort was 13 mo. In another report by Thomas *et al.*^[66], 17 (43%) of 40 patients achieved PFS at 16 wk, and the PFS rate at 24 wk was 28%. No PR or complete response was observed in this study. The median time to failure, defined as either disease progression or death, was 13.3 wk. The median time of OS was 25.0 wk (95%CI: 17.9-42.3) from the date of Erlotinib therapy initiation. In the Eastern Cooperative Oncology Group's E1203 study, Gefitinib provided at 250 mg daily was examined in a single-arm phase II study^[67]. A two-stage design was used, and 31 patients were accrued to the first stage. One patient had PR and seven patients had stable disease. The median PFS was 2.8 mo (95%CI: 1.5-3.9) and median OS was 6.5 mo (95%CI: 4.4-8.9). The criterion for second stage accrual was not met, and the authors concluded that gefitinib as a single agent was not active in advanced HCC. Lapatinib, a selective dual inhibitor of both EGFR and HER-2/NEU tyrosine kinases, also demonstrated modest activity in HCC. Among the 40 patients with advanced HCC, the response rate was 5%, PFS 2.3 (95%CI: 1.7-5.6) mo, and OS of 6.2 (95%CI: 5.1-infinity) mo^[68].

Monoclonal antibodies against EGFR

Cetuximab, a chimeric monoclonal antibody against EGFR, was tested in two phase II studies in patients

with advanced HCC. In a phase II study, 30 patients with advanced HCC were enrolled^[69]. The initial dose of cetuximab was 400 mg/m² provided intravenously, followed by weekly intravenous infusions at 250 mg/m². No responses were seen. Five patients had stable disease (median time, 4.2 mo; range, 2.8-4.2 mo). The median OS was 9.6 mo (95%CI: 4.3-12.1) and the median PFS was 1.4 mo (95%CI: 1.2-2.6). Cetuximab trough concentrations were not notably altered in patients with Child-Pugh A and B cirrhosis. The combination of cetuximab with gemcitabine and oxaliplatin (GEMOX) was evaluated in a phase II study. All patients received cetuximab at an initial dose of 400 mg/m² followed by 250 mg/m² weekly, gemcitabine 1000 mg/m² on day 1, and oxaliplatin at 100 mg/m² on day 2, repeated every 14 d until disease progression or limiting toxicity. Of the 45 patients enrolled, the confirmed response rate was 20% and disease stabilization rate was 40%. The median PFS and OS were 4.7 mo and 9.5 mo, respectively. The 1-year survival rate was 40%. Given the reported antitumor activity of GEMOX in prior phase II studies and the lack of activity of cetuximab as single agents, the relative contribution of cetuximab to this regimen remains to be defined^[70].

The combination of cetuximab with capecitabine and oxaliplatin was evaluated in a single-arm phase II study^[71]. Patients received capecitabine at 850 mg/m² twice daily for 14 d, oxaliplatin on day 1 at 130 mg/m² intravenously, and cetuximab at 400 mg/m² on day 1 followed by 250 mg/m² weekly in a 21-d cycle. Of the 25 patients enrolled, data for efficacy were available for 20 patients. Response rate was 10% (95%CI: 1-33), and TTP was 4.3 mo (95%CI: 2.3-5.0). Although most patients tolerated the treatment well, diarrheal and electrolyte abnormalities including hypomagnesemia and hypocalcemia were more pronounced in this population.

mTOR inhibitors

mTOR functions to regulate protein translation, angiogenesis, and cell-cycle progression in many cancers, including HCC. Preclinical data have demonstrated that mTOR inhibitors were effective in inhibiting cell growth and tumour vascularity in HCC cell lines and HCC tumour models. The importance of the mTOR pathway in HCC was examined in a comprehensive study with 314 HCC and 37 nontumoral tissues that used a series of molecular techniques to assess mutation, DNA copy number changes, messenger RNA and gene expression, and protein activation^[72]. Aberrant mTOR signalling (p-RPS6) was present in half of the cases and chromosomal gains in rapamycininsensitive companion of mTOR (RICTOR) (25% of patients), and positive p-RPS6 staining correlated with HCC recurrence after resection.

A number of mTOR inhibitors (sirolimus, temsirolimus, and everolimus) are available clinically. Retrospective studies in patients who underwent liver transplantation for HCC have shown that patients who received sirolimus for immunosuppression had a much

lower rate of tumour recurrence than those who received calcineurin inhibitors. Clinical studies with mTOR inhibitors alone and in combination with either targeted agents or chemotherapeutic agents in advanced HCC are at an early stage of clinical development. Chen *et al.*^[73] recently reported their early experience of a randomized phase I pharmacokinetic study of everolimus in advanced HCC. Two different schedules were tested: continuous daily dosing and once-weekly dosing. A total of 36 patients were enrolled. Dose-limiting toxicities observed included hyperbilirubinemia, high levels of alanine aminotransferase, thrombocytopenia, infection, diarrhea, and cardiac ischemia. The MTD for weekly and daily dosing schedules was determined to be 70 and 7.5 mg, respectively. Interestingly, reactivation of hepatitis B and C virus was observed in four and one patients, respectively. The disease control rate of 31 evaluable patients was 61% (10 of 16) and 46.7% (7 of 15, including one case of PR) of patients receiving daily and weekly treatment, respectively.

In patients with advanced HCC, everolimus produced a median PFS of 3.8 mo and a disease control rate of 44% in phase I / II testing. Consequently, a phase III EVOLVE-1 trial to compare everolimus with BSC in patients with HCC who progressed on or after sorafenib or who were intolerant to sorafenib, has been completed and everolimus did not show survival benefit for patients as announced in a press release on August 7 2013^[74].

MEK inhibitor

HCC is characterized by frequent MEK/ERK activation in the absence of RAS or RAF mutation. A multicenter, singlearm phase II study with a two-stage design was conducted with AZD6244, a specific inhibitor of MEK, in advanced HCC^[75]. The primary end point was response rate. AZD6244 was administered orally at a dose of 100 mg twice a day. Of the 19 patients enrolled, 16 had response data available. Despite the good tolerability of AZD6244, it showed minimal activity in advanced HCC. No response was observed, and stable disease was observed in 37.5% of the patients. The median TTP was only 8 wk (95%CI: 6.6-11.1).

Monoclonal antibodies against GPC-3

Glypican-3 (GPC-3) is a member of the glypican family, a group of heparan sulfate proteoglycans linked to the cell surface through a glycosylphosphatidylinositol (GPI) anchor. Glypicans play an important role in cell growth, differentiation, and migration^[76,77].

GPC-3 protein is expressed in a wide variety of tissues during development, but the expression in most adult tissues is suppressed by the methylation of DNA within its promoter region^[78]. Recently, it was shown that GPC-3 is highly expressed, both at the mRNA and protein level, in HCC^[79]. Immunohistochemical studies have shown that GPC-3 is expressed in approximately 70%-100% of surgically removed or biopsied HCC tissues, whereas it is not detectable in adjacent non-tumoral lesions^[80]. GPC-3 may promote hepatocellular carcinoma

growth by stimulating the canonical Wnt pathway and it also interacts with the IGFII-IGF1R pathway. Others have suggested that it may play a role in FGF signaling as well. GPC-3 therefore may represent a specific tumor marker and a potential target for therapy in HCC^[81].

GC33 (RO5137382) is a recombinant, humanized monoclonal antibody that binds to human GPC-3 with high affinity. The nonclinical pharmacological assessments have shown that GC33 elicits antibody dependent cellular cytotoxicity (ADCC) through human peripheral blood mononuclear cells as well as mouse effector cells against GPC-3-expressing human HCC and hepatoblastoma cell lines *in vitro*. It also showed anti-tumor activities in several mouse xenograft models inoculated with human HCC cell lines expressing GPC3.

Activity is proportional to cell surface expression of the target across 3 xenograft models, and is associated with macrophage infiltration into the xenografts. Direct activity of GC33 *in vitro* was not observed, suggesting that the relevant mechanism of action (MoA) is *via* ADCC. Two phase I studies are being conducted in the United States: GC-001US (GC33 monotherapy), and GC-002US (GC33/Sorafenib combination) and one study in Japan: GC-003JP (GC33 monotherapy). The dose escalation phase of GC-001US has completed accrual at planned doses up to and including 20 mg/kg per week. Is now ongoing a phase II trial, in second line setting, designed to establish the efficacy of GC33 compared to placebo in patients whose hepatocellular cancer tumor expresses the GPC-3 protein.

CONCLUSION

Despite decades of efforts by many investigators, no studies with systemic chemotherapy or hormone therapy have demonstrated improved survival in patients with advanced HCC. sorafenib has emerged as the new standard treatment for advanced HCC also patients with advanced HCC who failed first-line therapy could have substantially improved prognosis if they had Child-Pugh A liver reserves or were potentially eligible for clinical trials^[82].

Many molecularly targeted agents are at different stages of clinical development in HCC, and several agents, including Sunitinib, Brivanib, Tivantinib and Everolimus are being tested in phase III studies. Combining targeted agents that inhibit different pathways in hepatocarcinogenesis is an area of active investigation.

Future research should continue to unravel the mechanism of hepatocarcinogenesis and to identify key relevant molecular targets for therapeutic intervention. While we are developing other antiangiogenic and targeted agents in HCC, it is imperative that we continue our efforts to identify and validate surrogate and predictive biomarkers that would be helpful to predict clinical efficacy, toxicity, and resistance to these agents.

After decades of disappointing results for systemic treatment of HCC, exciting developments are expected in this once neglected field of clinical oncology research.

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Role of stereotactic body radiation therapy for hepatocellular carcinoma

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Abstract

The integration of new technologies has raised an interest in liver tumor radiotherapy, with literature evolving to support its efficacy. These advances, particularly stereotactic body radiation therapy (SBRT), have been critical in improving local control or potential cure in liver lesions not amenable to first-line surgical resection or radiofrequency ablation. Active investigation of SBRT, particularly for hepatocellular carcinoma (HCC), has recently started, yielding promising local control rates. In addition, data suggest a possibility that SBRT can be an alternative option for HCC unfit for other local therapies. However, information on optimal treatment indications, doses, and methods remains limited. In HCC, significant differences in patient characteristics and treatment availability exist by country. In addition, the prognosis of HCC is greatly influenced by underlying liver dysfunction and treatment itself in addition to tumor stage. Since they are closely linked to treatment approach, it is important to understand these differences in interpreting outcomes from various reports. Further studies are

required to validate and maximize the efficacy of SBRT by a large, multi-institutional setting.

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Key words: Hepatocellular carcinoma; Liver cirrhosis; Liver neoplasms; Radiation therapy; Stereotactic body radiation therapy

Core tip: The integration of new technologies has raised an interest in radiotherapy for hepatocellular carcinoma (HCC), with literature evolving to support its efficacy. These advances, particularly stereotactic body radiation therapy (SBRT), have been critical in improving local control or potential cure in liver lesions not amenable to first-line surgical resection or radiofrequency ablation. Active investigation of SBRT has recently started, yielding promising local control rates. However, information on optimal treatment indications, doses, and methods remains limited. In HCC, significant differences in patient characteristics and treatment availability exist by country. Further studies are required to validate and maximize the efficacy of SBRT.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is currently the fifth most common solid tumor worldwide and the third leading cause of cancer-related death^[1]. The definitive treatment for HCC has evolved primarily to be surgery, or-

thotopic liver transplantation, percutaneous ablation, or partial transarterial chemoembolization (TACE)^[2]. However, in some patients, these therapies are not feasible. This review describes the evolution of and current practices for radiation therapy, with particular focus on stereotactic body therapy (SBRT) in treating these types of tumors in patients who are not candidates for definitive treatment. We also discuss the emerging role of SBRT as well as current outcomes, toxicities, and pathological and radiological findings after SBRT.

EPIDEMIOLOGY

The incidence of HCC is increasing in several developed countries, such as European nations and the United States, while in areas such as Japan and Singapore, the incidence of HCC seems to have stabilized or even fallen slightly^[3,4]. The geographic variability in the incidence of HCC is largely explained by the distribution of hepatitis B and C viruses, and by the patterns of exposure to key risk factors in each population. The prevalence of such infections is being controlled by vaccination, which should also influence future trends in HCC occurrence. Heavy alcohol consumption, obesity, and diabetes are also risk factors and substantial causes of HCC in Europe and the United States.

The broad spectrum of HCC epidemiology and treatments is expected to affect prognosis. When referring to the literature on HCC treatment outcomes, it is important to carefully understand the differences in patient characteristics in each report, because these factors significantly affect outcomes as well as clinical application of various treatments.

STANDARD LOCAL TREATMENT OPTIONS FOR HCC

In HCC, prognosis is greatly influenced by underlying liver dysfunction and treatment itself, as well as tumor stage, while in other solid tumors, it is generally only related to tumor stage^[2]. Unlike other cancers, many staging systems are used for HCC, including the Barcelona Clinic Liver Cancer (BCLC) staging^[2], tumor-node-metastasis^[5], Okuda^[6], Cancer of the Liver Italian Program^[7], and Japan Integrated Staging^[8] scoring systems. Among these, the BCLC staging system considers the relevant parameters of all important dimensions and divides patients into very early/early, intermediate, advanced, and end-stage to recommend optimal treatment. Early-stage HCC patients are considered for potentially curative options such as resection, ablation, and transplantation. Patients with intermediate stage disease may benefit from TACE, whereas patients with advanced stage disease, or who cannot benefit from other options, are given sorafenib, an oral multikinase inhibitor, as the standard treatment.

Despite recent advances in early detection and diagnosis, only 30%-40% of patients with HCC may benefit from radical therapies. For patients who are not eligible

for these curative therapies, two randomized trials have shown improved survival using TACE compared with symptomatic therapy alone^[9,10]. However, TACE is somewhat controversial: a review from the Cochrane library that considered all randomized trials that compared TACE *vs* placebo, sham, or no intervention concluded that no firm evidence exists to support or refute TACE for patients with unresectable HCC^[11]. In addition, the local control rate for TACE is inferior to those of resection and percutaneous ablation (82%-98% at 3 years)^[12]. At best, the 3-year local control rate of superselective TACE was reported to be 65.3% in 123 patients with HCC < 5 cm in diameter^[13]. Nevertheless, patients who have limited tumor burden but are not suitable for radical therapies usually undergo TACE despite its relatively low efficacy. Improving the outcomes of these patients is one of the major challenges in HCC management.

DIFFERENCE IN TREATMENT APPROACH BY COUNTRIES

The differences in patient characteristics or treatment availability by country are closely linked to the treatment approaches used in each country. Currently, early HCC diagnosis is increasingly feasible in countries with wider implementation of surveillance policies, which enables the application of curative treatments. Applicability of standard local treatments varies according to geographic distribution, with 50%-70% of cases in Japan being suitable for curative treatment, compared to only 25%-40% of cases in Europe and the United States, and 10% in Africa^[14]. Once a high-risk cohort is identified, follow-up surveillance has contributed to early detection of HCC, although its efficacy appears to vary by country. In fact, in a recent Japanese cohort including 1432 patients, careful ultrasonography surveillance performed by highly skilled operators resulted in the average size of detected tumors being 1.6 cm \pm 0.6 cm, with < 2% of the cases exceeding 3 cm^[15].

Treatment differences by country are also prominent for organ transplantation. Orthotopic liver transplantation offers the best chance for cure, particularly in patients with decompensated liver disease. Excellent results can be achieved in patients with solitary HCC < 5 cm, or up to three nodules < 3 cm, and without extrahepatic or vascular spread, known as the Milan criteria^[16]. However, the chance of transplantation is extremely limited in many countries due to the lack of sufficient liver donors. Furthermore, in contrast to western countries, Asia has cultural and religious barriers to organ donation from deceased individuals. The number of deceased donors per million population ranges from 0.07-6.5 among Asian transplantation centers, which is far below those of western countries (*e.g.*, 35.1 per million population in Spain and 25.2 per million population in the United States)^[17].

RADIATION THERAPY FOR HCC

Historically, treatment with conventionally fractionated

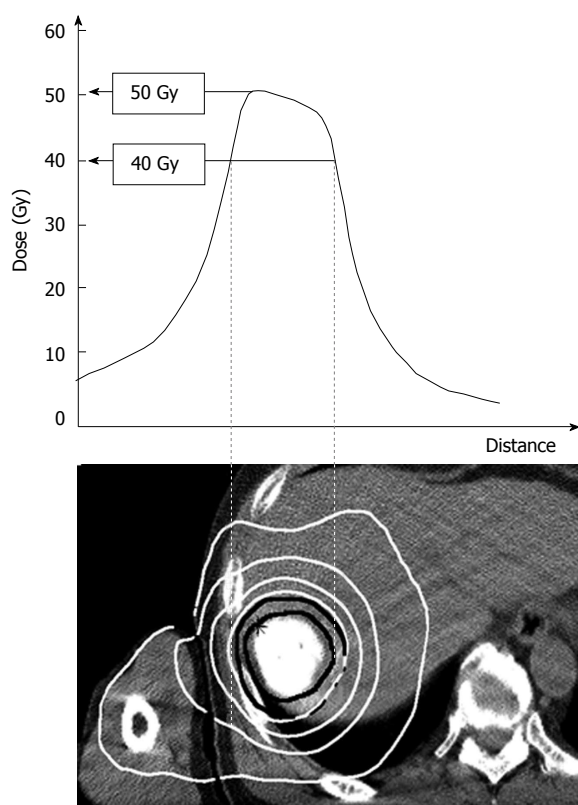


Figure 1 Dose distribution of stereotactic body radiation therapy for hepatocellular carcinoma at a dose of 40 Gy in 5 fractions, prescribed at the periphery of the target volume. The isodose lines (white solid lines) from inner to outer represent 40, 30, 20 and 10 Gy, respectively. The center of the tumor receives as high as 125% of the prescribed dose.

(1.8-2 Gy/fraction over several weeks) and 2-dimensionally-planned radiation is associated with high rates of local progression and short median survival duration. While a radiation dose-response has been observed in unresectable HCC, the delivery of high doses of radiation using conventional techniques has been limited by hepatotoxicity. Given these limitations, radiation therapy is usually not curative. Compared to other local therapies, the clinical data supporting evidence for radiotherapy in HCC patients are extremely limited^[18].

As Dr. Dawson^[19] descriptively discussed regarding how radiation therapy fit into the spectrum of liver cancer local therapies, HCC patients suitable for focal irradiation extend from very early to intermediate BCLC stage. For those who are unsuitable for resection, transplantation, or ablation, definitive radiotherapy or radiotherapy can be administered as a bridge to transplantation^[20-22]. Definitive radiotherapy is also considered for those patients who are unsuitable for or refractory to TACE. For those patients who have portal invasion, systemic chemotherapy (*e.g.*, sorafenib) can be added to definitive radiotherapy.

SBRT

Numerous advances in external-beam radiation therapy allow for more accurate targeting, and make aggressive dose-fractionation strategies possible using techniques such as SBRT. Originally developed for the treatment of

Table 1 Eligibility criteria for different treatment modalities

	Surgery	Percutaneous ablative therapy	TACE	SBRT
Tumor size	< 5 cm (or more)	< 3 cm	> 3-5 cm	4 (or 5) cm
Number of tumors	< 3	Depends on location	1-multiple (> 4)	< 1-3
Location or characteristics	Depends on liver function	Away from large vessels or biliary system	Hypervascular lesions	Away from bowels
Local control (2 yr)	> 90%	> 90%	< 65%	> 90%
Level of evidence	High	Intermediate-high	Intermediate-high	Low
Invasiveness	High	Less	Less	None
Damage to the liver	High	Low	Low-moderate	Low-moderate

SBRT: Stereotactic body radiation therapy; TACE: Transarterial chemoembolization.

intracranial malignancies (*i.e.*, radiosurgery), SBRT has since been adopted for the treatment of extracranial diseases. For example, SBRT has been shown to be a highly effective and well-tolerated treatment in patients with medically inoperable or high-risk operable stage I non-small cell lung cancer^[23].

The use of SBRT for liver malignancies was pioneered by Dr. Blomgren *et al.*^[24] at the Karolinska Institute, Stockholm in the early 1990s. SBRT refers to the use of stereotactic non-coplanar conformal radiation therapy intended for a small number of significantly larger fraction sizes (usually 8-12 Gy/fraction), while limiting the dose to adjacent normal tissues. The steep dose gradient within the target volume leads to tight conformity with steep and isotropic dose fall-off and high dose delivery to the target volume (Figure 1).

SBRT should be implemented with accurate patient repositioning, target localization, and control of breathing-related motion by breathing control devices such as abdominal compression, gating, and tracking systems, as well as some form of image-guided radiation therapy (IGRT) to improve set-up accuracy and treatment delivery^[25].

INDICATIONS FOR SBRT

Although the indications for SBRT for hepatic malignancy have evolved, the role of SBRT in HCC is less clear. Future studies should focus not only on maximizing efficacy, but also on determining how SBRT should be used in the context of other previously established therapies. Careful patient selection is required and SBRT should be considered only after thorough discussion within a multi-disciplinary team, with all legitimate treatment options also considered.

Eligibility criteria for different treatment techniques are outlined in Table 1. In general, SBRT and other local therapies can complementarily divide the roles between

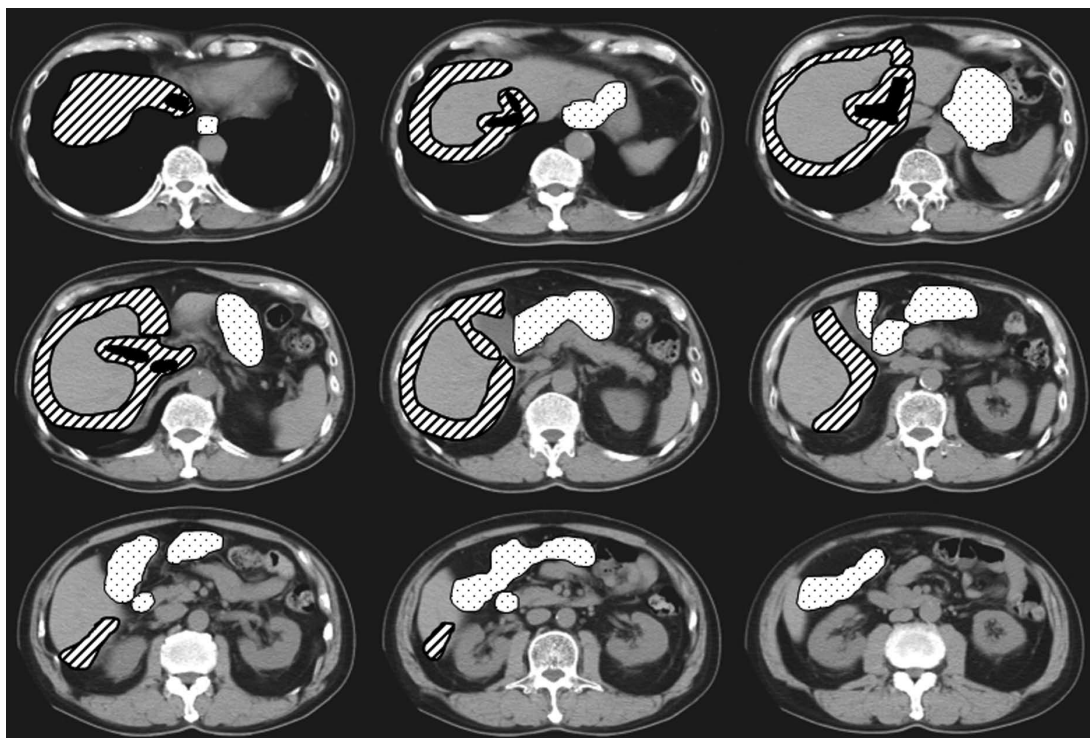


Figure 2 Computed tomography sections demonstrating typical locations treated with stereotactic body radiation therapy. Areas indicated with hatched lines can be safely treated with stereotactic body radiation therapy (SBRT), although these areas are difficult to approach for percutaneous ablation. Liver tumors located adjacent to the stomach and bowels (dotted areas) are not suitable for SBRT. Other unmarked areas can be treated either with SBRT or percutaneous ablation.

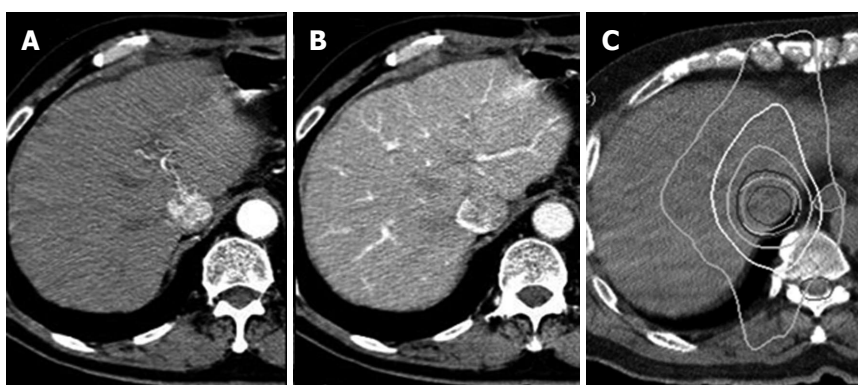


Figure 3 Hepatocellular carcinoma case that could not be effectively or safely treated with any treatment except for stereotactic body radiation therapy. The tumor invading the vena cava is enhanced in arterial phase and shows a defect in portal phase on dynamic computed tomography (A and B). An axial view of radiation dose distribution. The isodose lines (white lines) from inner to outer represent 40, 30, 20, and 10 Gy, respectively (C).

each modality. SBRT is feasible even for lesions that are not eligible for surgery or percutaneous ablation. For example, patients whose lesions are located in a central portal area or regions adjacent to great vessels or the biliary system are good candidate for SBRT^[26]. In addition, lesions located just below the diaphragm or at the surface of the liver are also excellent targets for SBRT. Figure 2 illustrates typical liver locations for which SBRT can be safely delivered. SBRT is difficult to perform for lesions near the bowels due to the risk of gastrointestinal perforation, bleeding, and ulcer. Examples of patients who could not be treated with other local therapies but received SBRT are shown in Figures 3-5.

There is always a waiting period between listing and transplantation, and this varies between institutions. Many therapies have been used as a “bridge” to transplantation, and SBRT has also been evaluated as a means to bridge to transplantation. As a bridging therapy, SBRT has been reported to be feasible and well tolerated^[20,21,27]. Furthermore, it enables patients to remain on the list for frequently curative transplantation while waiting for donated livers to become available.

SBRT OUTCOMES AND OPTIMAL DOSES

Outcomes of SBRT for HCC are summarized in Table

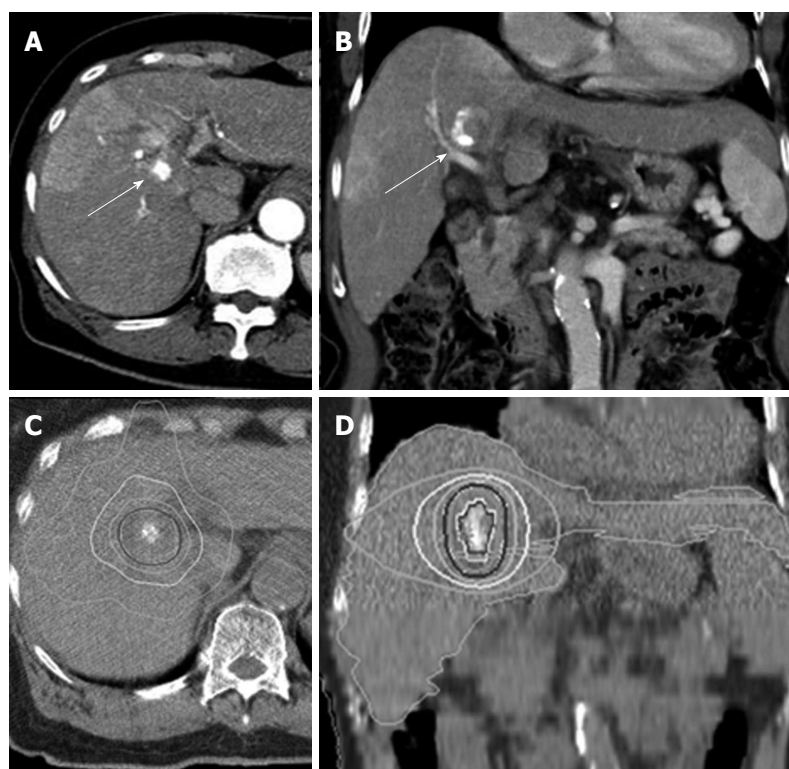


Figure 4 Case of hepatocellular carcinoma located in the hepatic hilum. Surgical resection would have required a right lobectomy. Percutaneous ablative therapy was impossible due to involvement of the biliary system and large vessels near the tumor. Axial and coronal views of a tumor with partial lipiodol deposit (A and B, arrows). Axial and coronal views of radiation dose distribution (C and D). The isodose lines (white lines) from inner to outer represent 40, 30, 20, and 10 Gy, respectively.

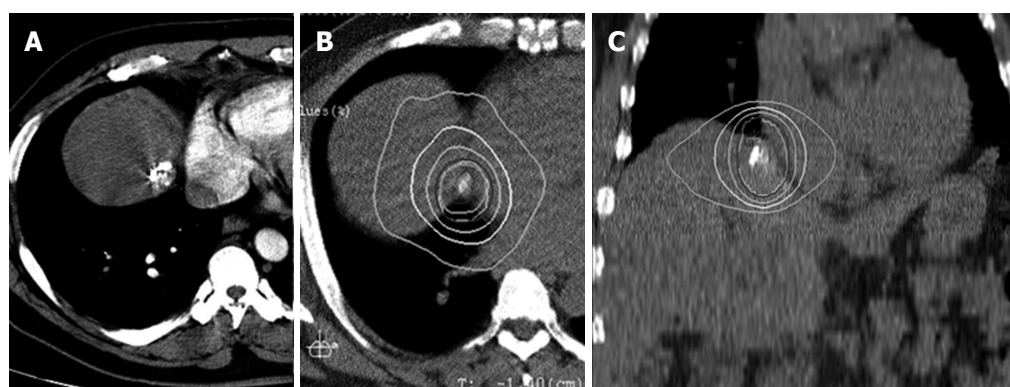


Figure 5 Case of hepatocellular carcinoma. Located adjacent to the right atrium (A). Axial and coronal view of radiation dose distribution (B and C). The isodose lines (white lines) from inner to outer represent 40, 30, 20, and 10 Gy, respectively.

2^[28-43]. A total of four prospective studies that exclusively evaluated HCC, as well as other retrospective studies demonstrated promising treatment effects (Table 3). The number of reports of successful SBRT studies had been increasing since 2006. Earlier studies involving SBRT for liver tumors included not only HCC but also cholangiocarcinoma and metastatic liver tumors, which made it difficult to compare the results between studies. Although the literature for SBRT is primarily composed of retrospective, small, single-institution series, SBRT has been associated with high local control rates, mostly in the range of 70%-90% at 1-2 years.

Various prescribed doses and treatment planning strategies are presently employed by different groups^[28-43], and information about optimal treatment doses remains limited. Some studies employed SBRT alone, while others combined TACE as part of the treatment. Tumor size varied

from 2-3 cm to approximately 5-7 cm. These differences are attributed to the geographic variability in HCC etiology and treatment availability, as previously mentioned. Therefore, the prescribed doses are expected to vary between studies even if only SBRT is used.

In general, fixed doses are employed for relatively small tumors with a median diameter of approximately 3 cm, *e.g.*, 36 Gy/3 fractions or 40 Gy/5 fractions (Table 2). In contrast, modified doses are employed for relatively larger targets according to normal liver tolerance depending on tumor size and normal liver volume. Using normal tissue complication probability models^[44], prescribed doses can be prospectively assigned while maintaining the same estimated risk of liver complication. With this approach, an iso-toxic SBRT regimen was developed at Princess Margaret Hospital at the University of Toronto^[45]. The dose per fraction was determined based on

Table 2 Prospective studies of stereotactic body radiation therapy for hepatocellular carcinoma and other liver tumors

Ref.	Country	Patient number	Median volume, mL	Median size, cm	Median dose (range)/fraction, Gy	Median follow-up (range), mo	Local control	Overall survival
Cárdenes <i>et al</i> ^[29]	United States (Indiana)	17	34 (8-95)	-	Variable CP-A: 36-48 Gy/3 fr CP-B: 40 Gy/5 fr	24 (10-42)	100%	75% (1 yr) 60% (2 yr)
Andolino <i>et al</i> ^[37]	United States (Indiana)	60	29 (2-112)	3.2 (1-6.5)	Fixed CP-A: 44 Gy/3 fr CP-B: 40 Gy/5 fr	27 (2-52)	90% (2 yr)	67% (2 yr)
Bujold <i>et al</i> ^[30]	Canada	102	117 (1-1913)	7.2 (1.4-23.1)	Variable 36 (24-54) Gy/6 fr	31 (2-36)	87% (1 yr)	Median 17 mo
Kang <i>et al</i> ^[31]	South Korea (Korea Inst. of Radiological and Medical Sciences)	47	15 (2-213)	2.9 (1.3-7.8)	57 (42-60) Gy/3 fr	17 (6-38)	95% (2 yr)	69% (2 yr)

CP: Child-Pugh.

Table 3 Retrospective studies of stereotactic body radiation therapy for hepatocellular carcinoma and other liver tumors

Ref.	Country	Patient number	Median volume (mL)	Median size, cm	Median dose (range)/fraction, Gy	Median follow-up (range) (mo)	Local control	Overall survival
Choi <i>et al</i> ^[32] , 2006	South Korea (The Catholic Univ. of Korea)	20	-	3.8 (2-6.5)	Fixed 50 Gy/5 or 10 fr	23 (3-55)	100%	70% (1 yr) 43% (2 yr)
Zhang <i>et al</i> ^[72] , 2007	China (Hebei)	27	-	< 5 cm: 18% 3-5 cm: 41% > 5 cm: 41%	Median 40 (32-42) Gy/5 fr	10 (3-21)	22% (1 yr)	-
Choi <i>et al</i> ^[33] , 2008	South Korea (The Catholic Univ. Korea)	31	25 (4-57)	-	Variable 33 (30-39) Gy/3 fr	11 (2-19)	100%	81% (1 yr)
Louis <i>et al</i> ^[34] , 2010	Belgium	25	48 (7-363)	-	Fixed 45 Gy/3 fr	13 (1-24)	95% (1 yr)	79% (1 yr) 52% (2 yr)
Kwon <i>et al</i> ^[35] , 2010	South Korea (The Catholic Univ. of Korea)	42	15 (3-82)	-	Variable 30-39 Gy/3fr	29 (8-4)	72% (1 yr) 68% (3 yr)	93% (1 yr) 59% (3 yr)
Seo <i>et al</i> ^[36] , 2010	South Korea (Korea Inst. of Radiological and Medical Sciences)	38	41 (11-464)	-	Variable 33-57 Gy/3-4 fr	15 (3-27)	66% (2 yr)	61% (2 yr)
Huang <i>et al</i> ^[38] , 2012	Taiwan	36	-	4.4 (1.1-12)	Variable 37 (25-48 Gy)/4-5 fr	14 (2-35)	88% (1 yr) 75% (2 yr)	64% (2 yr)
Honda <i>et al</i> ^[39] , 2012	Japan (Hiroshima)	30	-	1.6 (1-3)	Fixed 48 Gy/4 fr or 60 Gy/8 fr	12 (6-38)	100%	100% (1 yr) 100% (3 yr)
Bae <i>et al</i> ^[40] , 2013	South Korea (Korea Inst. of Radiological and Medical Sciences)	35	131 (21-2189)	-	Variable 45 (30-60) Gy/3-5 fr	14 (1-44)	69% (1 yr) 51% (3 yr)	52% (1 yr) 21% (2 yr)
Xi <i>et al</i> ^[41] , 2013	China (Sun Yat-sen Univ.)	41	65 (± 48)	-	Variable 36 (30-48) Gy/6 fr	10 (4-25)	95%	50% (1 yr)
Sanuki <i>et al</i> ^[43] , 2013	Japan (Ofuna)	185	8 (1.5-65)	-	Fixed CP-A: 40 Gy/5 fr/ CP-B: 35 Gy/5 fr/	24 (3-80)	91% (3 yr)	70% (3 yr)

CP: Child-Pugh.

Table 4 Studies of liver tumors including hepatocellular carcinoma, cholangiocarcinoma, and liver metastasis

Ref.	Country	Study design	Tumors (patient number)	Median volume (mL)	Median size (cm)	Median dose (range)/fraction, Gy	Median follow-up (range) (mo)	Local control	Overall survival
Blomgren <i>et al</i> ^[24] , 1995	Sweden	Retrospective	HCC+CCC/ metastasis (20/21)	22	-	Fixed 30 Gy/2-3 fr	11	HCC + CCC: 100% Metastasis: 95%	-
Tse <i>et al</i> ^[28] , 2008	Canada	Phase I	HCC/CCC (31/10)	173 (9-1913)	-	Variable Median 36 (24-54) Gy/6 fr	18 (11-39)	65% (1 yr)	48% (1 yr)
Herfarth <i>et al</i> ^[47] , 2001	Germany	Phase I - II	HCC/CCC (4/54)	10 (1-132)	-	Dose escalation 14-26 Gy/1 fr	6 (1-26)	81% (18 mo)	-
Wulf <i>et al</i> ^[48] , 2006	Switzerland	Prospective	HCC + CCC/ metastasis (5/51)	HCC+CCC: 14-516 Metastasis: 9-355	-	Variable Low dose: 30 Gy/3 fr or 28 Gy/4 fr High dose: 36-38 Gy/3 fr or 26 Gy/1 fr	HCC + CCC: 15 (2-48) Metastasis: 15 (2-85)	HCC + CCC: 100% Metastasis: 99% (1 yr), 66% (2 yr)	72% (1 yr) 32% (2 yr)
Méndez-Romero <i>et al</i> ^[49] , 2006	The Netherlands	Retrospective	HCC/ metastasis (11/34)	22 (10-322)	3.2 (0.5-7.2)	No cirrhosis and ≥ 4 cm: 37.5 Gy/3 fr Cirrhosis and < 4 cm: 25 Gy/5 fr or 30 Gy/3 fr	13 (0.5-31)	94% (1 yr) 82% (2 yr)	HCC: 75% (1 yr), 40% (2 yr) Metastasis: 82% (1 yr), 54% (2 yr)
Iwata <i>et al</i> ^[50] , 2010	Japan (Nagoya City University)	Retrospective	HCC/ metastasis (6/12)	-	2.3 (1.2-3.5)	Variable 50 or 55 Gy/10 fr	15	86% (1 yr)	94% (1 yr)
Goodman <i>et al</i> ^[51] , 2010	United States (Memorial Sloan Kettering Cancer Center)	Phase I	HCC/ metastasis (2/24)	33 (0.8-147)	-	Dose escalation 18-30 Gy/1 fr	17 (2-55)	77% (1 yr)	50% (2 yr)
Dewas <i>et al</i> ^[53] , 2012	France	Retrospective	HCC + CCC/ metastasis (54/99)	32 (0.2-500)	3.3 (0.5-11)	Variable 45 Gy/3 fr	15 (12-18)	84% (1 yr) 75% (2 yr)	-
Ibarra <i>et al</i> ^[54] , 2012	United States (Cleveland)	Retrospective, multicenter	HCC/CCC (21/11)	HCC: 334 (10-1914) CCC: 80 (31-819)	-	HCC: 22 (18-26) Gy/1 fr CCC: 30 (22-30) Gy/1 fr	13 (0.5-54)	84% (1 yr) 75% (2 yr)	87% (1 yr) 55% (2 yr)

CCC: Cholangiocarcinoma; HCC: Hepatocellular carcinoma.

the effective volume of normal liver irradiated (Veff). On a 6-fraction schedule, when the Veff was low (< 25%), doses of 54 Gy (9 Gy × 6) were delivered. For patients with a high Veff (25%–60%), doses from 30–45 Gy (5 to 7.5 Gy × 6) were delivered. In their phase I study of 102 HCC patients, the majority of whom had portal vein tumor thrombosis, the 1-year local control rate was 87%^[30]. Univariate analysis revealed that higher SBRT doses were associated with higher local control (HR = 0.96; *P* = 0.02).

Both fixed-dose and variable-dose prescription approaches have their own rationale, and it is important to understand the differences in treatment intention (curative or semi-radical) and objectives (early or advanced). Two potential concepts may define the prescribed radiation dose: one is to deliver the maximum dose if dose constraints to the organs at risk are satisfied (the maximum

tolerable dose); the other is to administer the necessary minimum dose with sufficient efficacy (the minimum effective dose, or, the ALARA: as low as reasonably achievable principal). The former concept appears to be suitable for larger tumors to maximize antitumor effects. In contrast, the latter concept may be reasonable for small HCCs, because intrahepatic recurrences frequently occur after treatment (68% in 5 years)^[46] and they are repeatedly treated while underlying cirrhosis progressively develops over time.

It is also important to note that many reports include cholangiocarcinoma or metastatic liver tumors (Table 4); therefore, it is difficult to compare their survival with those who underwent resection and ablation^[24,45,47-54]. While patients with liver metastatic disease have relatively normal liver function and tolerate radiation, patients with

HCC have pre-existing liver dysfunction, and radiation tolerance is less well established. In addition, radiosensitivity of these tumors appears to be different^[55]. While metastatic lung tumors (particularly those from colorectal cancer) are reported to require dose escalation due to relatively low radiosensitivity^[56], increasing the dose for HCC tumors may not be necessary. In fact, in a study of 185 patients with HCC (median diameter, 27 mm) treated with SBRT of 35 Gy or 40 Gy in 5 fractions, both local control (91% and 89%, respectively; Log-rank $P = 0.99$) and overall survival (66% and 72%, respectively; $P = 0.54$) rates were equivalent between the two dose groups^[43].

Other factors may also affect treatment outcomes. Some reports on SBRT for HCC use TACE as a part of their treatment, or for validation of tumor location in each treatment session by visualizing the tumor with lipiodol on computed tomography (CT), while other reports treat patients with SBRT alone. In addition, since patients in most of the series were previously treated by other standard therapies, outcomes of these patients are much worse than those in whom surgery is performed as the first treatment. While achieving high local control rates (approximately 90%-100% in 2 years) with SBRT, the 2-year overall survival rates, which range from 52%-69%, seem to be compromised, most likely due to the inclusion of large tumors or heavily pretreated patients with repeated recurrences. In a retrospective analysis of 63 patients who had previously untreated HCC with a median tumor size of 2.6 cm, SBRT delivered 35-40 Gy in 5 fractions yielded 2- and 3-year local control rates of 95% and 92%, respectively, with a median follow-up duration of 31.1 mo^[57]. In this study, the overall survival rate was 73% in 3 years, which was comparable to outcomes treated with surgery or percutaneous ablation, considering these candidates were medically unfit for radical therapies. In the Japanese Nationwide Survey, the 3-year overall survival rates of patients with solitary tumors ≤ 2 cm and 2-5 cm treated with resection were 83%-90% and 70%-81%, respectively. Those treated with percutaneous ablation were 82%-88% and 66%-82%, respectively^[58]. According to these results, it is indicated that a high local control rate for SBRT similar to other standard local therapies can achieve equivalent overall survival.

TOXICITIES AFTER SBRT

Radiation-induced liver disease (RILD) is a dose-limiting complication of liver irradiation. This could be an important issue, particularly in patients with HCC, primarily in the context of underlying liver cirrhosis. Originally, RILD was thought to involve anicteric hepatomegaly, ascites, and elevated alkaline phosphatase typically occurring 2-12 mo after therapy. This endpoint can occur in patients who have otherwise fairly well functioning pretreatment livers and can be fatal once it occurs ("classic" RILD)^[59]. Caution must be exercised in patients with HCC derived from pre-existing liver disease, because patients with more severe liver disease are significantly less likely to

tolerate radiation^[60], which can manifest as "nonclassic" RILD. A review article by Pan *et al.*^[59] referred to nonclassic RILD as \geq Grade 3 elevated liver transaminases or worsening of Child-Pugh score by ≥ 2 . However, information about nonclassic RILD remains limited, and the clinical significance of such liver toxicities has not been validated, particularly for hypofractionated SBRT.

In general, SBRT can be performed safely. In sequential phase I and II trials of SBRT of 24 to 54 Gy in 6 fractions for 102 locally advanced HCCs, \geq Grade 3 toxicity was observed in 30% of patients. In these trials, there were seven deaths (7%) possibly related to treatment (1.1-7.7 mo after SBRT)^[28]. In a large retrospective study of 185 HCC patients treated with SBRT of 35 or 40 Gy in 5 fractions, acute but transient \geq Grade 3 toxicities were observed in 24 (13.0%) patients, and grade 5 liver failure occurred in 2 patients (1.3%)^[43].

Another major concern of SBRT-induced complications involves gastrointestinal toxicity. Gastric or duodenal ulcer or perforation has been reported^[38,40]. Such toxicities can be avoided when the target is approximately > 2 cm from the bowels. If the target is less than that distance, the dose or fraction size that can be delivered safely to the target often needs to be decreased to respect the radiation tolerance. In contrast, it appears that SBRT can be safely delivered to tumors located within or near the biliary system^[26].

PATHOLOGICAL AND RADIOLOGICAL FEATURES OF HCC AND NORMAL LIVER AFTER SBRT

Effect of SBRT to normal liver parenchyma

Normal liver changes after high-dose irradiation have been recognized to have the histopathologic features of veno-occlusive disease (VOD)^[61,62]. Olsen *et al.*^[22] reported on the histopathologic features underlying focal liver reactions to irradiation in 2 patients who underwent surgical resection following SBRT. They identified areas of radiation injury as having histologic characteristic of focal VOD with centrilobular congestion and fibrosis. These distinct areas were observed with clear demarcation between the irradiated and nonirradiated liver.

Radiographic normal tissue changes caused by irradiation have been described after SBRT in the absence of clinical manifestations of RILD. Most notable is a well-demarcated focal hypodensity of liver parenchyma that appears in the first few months after SBRT on CT, often referred to as focal liver reaction to radiation^[22,63-65] (Figure 6). They typically present as sharply demarcated areas from the surrounding liver tissue, which presents, often enhanced, in the portal-venous or late phases. According to a report on radiation-induced focal liver reactions in cirrhotic livers evaluated on dynamic CT, it began at a median of 3 mo, peaked at 6 mo, and disappeared about 9 mo later, and these appearances remained for more than 12 mo in at least one-third of patients^[64]. It is im-

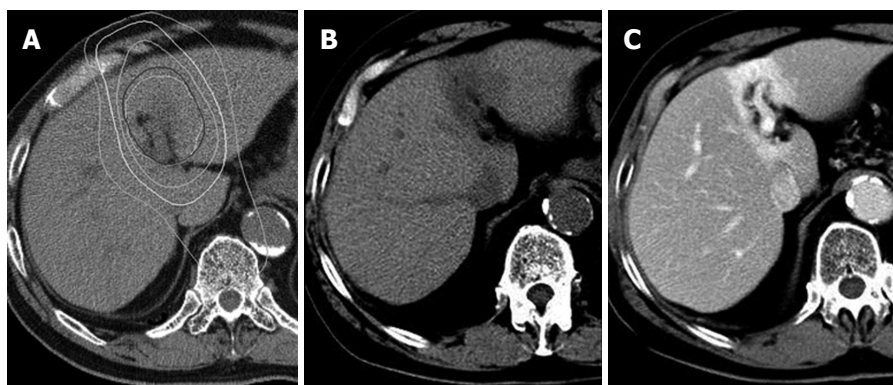


Figure 6 Typical focal liver reaction 3 mo after stereotactic body radiation therapy seen on computed tomography. An axial view of radiation dose distribution (A). The isodose lines (white lines) from inner to outer represent 40, 30, 20, and 10 Gy, respectively. Pre-enhancement computed tomography shows a low-density lesion corresponding to a high-dose area (B). A well-demarcated enhancement due to contrast retention indicating congestion is seen in portal phase (C).

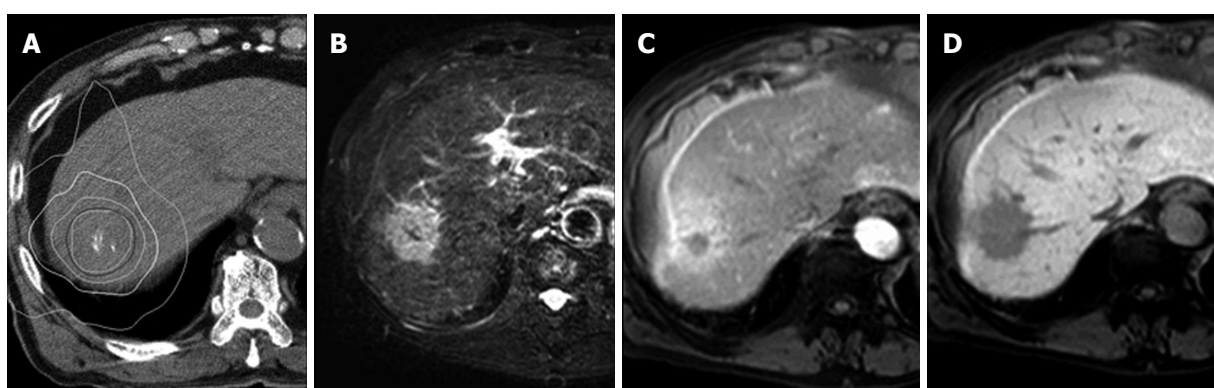


Figure 7 Typical focal liver reaction 4 mo after stereotactic body radiation therapy seen on gadoxetate acid-enhanced magnetic resonance imaging. An axial view of radiation dose distribution (A). The isodose lines (white lines) from inner to outer represent 40, 30, 20, and 10 Gy, respectively. A T2-weighted image shows a high-intensity area corresponding to a high-dose area (B), which is seen as an enhanced area in early phase after injection of gadoxetate acid (C). The hepatobiliary phase shows a well-demarcated low-intensity area (D).

portant that this type of reaction following SBRT to the liver is recognized, and it should not be misinterpreted as local recurrence, because the duration of tumor viability after SBRT overlaps with this time period^[63,65].

Focal liver reaction has primarily been evaluated using CT, and it can also be recognized on magnetic resonance imaging (MRI). The role of the contrast agent gadoxetate acid (Gd-EOB-DTPA) for MRI in the detection and characterization of HCC has been an area of active laboratory and clinical research^[66,67]. After intravenous injection, Gd-EOB-DTPA is gradually taken up by hepatocytes and is eventually excreted *via* the biliary pathway. Hepatocyte-phase Gd-EOB-DTPA-enhanced MRI can be used for the detection or characterization of hepatic lesions and potentially for the measurement of hepatocyte function. Figure 7 shows a clear demarcated focal liver reaction corresponding to a highly-irradiated area, seen as low signal intensity of adjacent liver parenchyma typically observed 1-6 mo after treatment in hepatobiliary phase images (15-20 min after Gd-EOB-DTPA injection).

Effect of SBRT to tumor

SBRT as a bridging therapy to orthotopic liver trans-

plantation provided an opportunity to study the histopathologic features of tumors treated with SBRT^[20,21,27]. According to these studies, this therapy can achieve a complete response rate of 27%. In contrast, most SBRT series reported a local control rate of 70%-100%, which implies that the effect of SBRT takes a considerable time to cause tumor cell death. Such discrepancy appears to be attributable to the difference in evaluation time; the interval between bridging SBRT to liver transplantation was approximately 4-7 mo while radiological response rate evaluation was performed at a median follow-up of approximately 12-24 mo. In fact, a retrospective study described the long-term imaging appearance of 42 small hypervascular HCCs following SBRT^[68]. In this study, the complete response rate increased from 24% ($n = 10$) at 3 mo to 67% ($n = 28$) and 71% ($n = 30$) at 6 and 12 mo, respectively. The 2-year local control rate was 97% and the overall complete response rate at maximum follow-up was 93% ($n = 39$), yet three enhanced tumors persisted for more than 2 years without evidence of progression. Cautious and continuous observation until tumor regrowth is required to evaluate the true effect of this treatment.

FUTURE RESEARCH DIRECTIONS

The most common site of first recurrence was the liver outside the irradiated volume, providing the rationale for studies combining regional or systemic therapies with SBRT. The Radiation Therapy Oncology Group (RTOG) has initiated a phase III study of sorafenib *vs* SBRT followed by sorafenib in HCC (ClinicalTrials.gov identifier, NCT01730937). Eligible patients have locally advanced HCC that is unsuitable for resection, transplantation, or radiofrequency ablation, or is unsuitable for TACE or refractory to TACE (BCLC Intermediate [B] or Advanced [C]). Dozens of additional clinical trials utilizing SBRT for HCC are being conducted around the world, suggesting that it is a promising and actively investigated treatment option. Among these studies, a Japanese multicenter group is conducting a study of SBRT for previously untreated solitary HCC patients who are unfit for resection or ablation (primarily BCLC stage 0 and A). The results should provide new information about the effect of SBRT as an alternative option for early HCC.

In addition to conformal photon external-beam delivery, data suggest improved outcomes with proton or charged-particle therapies^[69-71], particularly for large tumors. The optimal indication needs to be defined for SBRT and particle-beam therapies to separate the specific roles for each modality.

While the early outcomes of SBRT use in unresectable HCC are encouraging, further studies are required to validate these favorable results. A large, multi-institutional phase II study should be performed to evaluate the efficacy and toxicity of SBRT for unresectable HCC, as well as the feasibility of this treatment in a multi-institutional setting.

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Prognostic biomarkers for prediction of recurrence of hepatocellular carcinoma: Current status and future prospects

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Abstract

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer death worldwide, with region specific etiologies. Despite improvements made in the diagnosis of HCC, the prognosis of HCC patients remains poor due to the high recurrence rate of HCC. There is an urgent need for development of prognostic biomarkers to predict the risk of recurrence in HCC patients after "curative" treatment. Such stratification may aid in patient management and development of personalized medicine for HCC treatment. Omics based studies facilitate the study of global changes in biomolecules in a disease in a high throughput manner, and hence are well poised to understand the complex changes which led to HCC recurrence. The quantitative nature of data obtained from omics based studies allow for development of prognostic biomarkers based on changes in gene, protein and metabolite expression. In this review, we surveyed the ap-

plication of transcriptomics, proteomics and metabolomics in the study of HCC recurrence. We summarised the data in the literature from these three fields of studies that claimed to be prognostic for HCC recurrence. We critiqued on the limitations of each area of research and the challenges faced in translating the research results for clinical application in predicting HCC recurrence.

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Key words: Liver cancer; Biomarker; Transcriptomics; Proteomics; Metabolomics; Recurrence

Core tip: Hepatocellular carcinoma (HCC) patients have poor prognosis, largely due to the high incidence of recurrence. There is an urgent need for prognostic biomarkers to stratify patients with higher risk of recurrence to aid in clinical management. This review surveys the use of transcriptomics, proteomics and metabolomics in identifying prognostic biomarkers for HCC recurrence. Integration of data from various omics field allow for understanding of major pathways that were dysregulated in HCC recurrence, which could pave the way for development of personalized medicine for management of HCC recurrence.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most com-

mon cancer and third leading cause of cancer related deaths worldwide, with about 667000 new cases per annum^[1]. HCC usually develops on patients with liver cirrhosis, and the causes are region specific. Etiological factors of HCC include chronic hepatitis B and C viral infection, chronic alcohol consumption and consumption of aflatoxin-B1 contaminated food. Diagnosis of HCC has improved significantly, from the study of histological features of the tissue to the use of radiography, such as magnetic resonance imaging (MRI) and computed tomography (CT)^[2]. The improvements allow for earlier diagnosis of HCC, which opens up treatment options to the patients. Currently, liver transplantation and resection remains as the only curative treatment for HCC. However, about 60% of the patients suffered from HCC relapse even after curative resection, thus resulting in a poor prognosis for HCC^[3]. Hence, it is imperative to look for prognostic markers that could predict the risk of recurrence in HCC patients so that these patients could benefit from increased surveillance which could increase overall survival rates. Understanding the molecular mechanism behind HCC recurrence could lead to the development of novel therapeutics that could potentially be applied as palliative treatments for recurrent cancers.

Differences in etiology of early and late recurrence

It is widely believed that there are two major types of HCC recurrence^[4]. Dissemination of the remnant tumour cells after surgical resection results in recurrent tumours at other parts of the liver. Recurrence of this type usually peaks within two years after resection (early recurrence). Clinicopathological features associated with early recurrence include increased tumour size, presence of venous invasion, increased serum alpha-fetoprotein (AFP) protein and mRNA concentrations^[3,5-8]. The cirrhotic liver may also act as a “field” for tumour development. The diseased liver undergoes further molecular changes under the influence of factors such as hepatitis virus infection to form dysplastic nodules, which would lead to formation of *de novo* tumours, resulting in recurrence. The recurrent tumours are usually independent from the primary tumours, and tumours of this type peak at three years after resection (late recurrence). The biological and clinical differences between these two types of recurrence models leads to differences in research approaches, with the primary tumour tissues commonly used for studies on early recurrence, and surrounding non-tumour tissues as the samples for late recurrence.

Prognostic values of known HCC oncogenes and tumour suppressors

The prognostic values of oncogenes and tumour suppressors implicated in HCC carcinogenesis had been mixed. High nuclear β -catenin^[9] and elevated c-Myc expression^[10] were correlated with vascular invasion and shorter recurrence free survival (RFS), suggesting the involvement of Wnt signaling activation in promoting HCC recurrence. The combination of three factors, namely phosphatase and tensin homolog downregulation,

overexpression of p53 and increase in proliferating cell nuclear antigen labelling index, was associated with lower disease specific survival and early tumour recurrence^[11]. Hotspot mutations of p53 (R249S and V157F) were associated with a stem cell phenotype, which is linked to a poor overall survival (OS) in HCC patients^[12]. Interestingly, mutations in p53 and DNA mismatch repair protein Human mutS homolog 2 (hMSH2) had been linked to intrahepatic metastasis, as opposed to late recurrence^[13]. Mutations in p53 and hMSH2 might confer a more aggressive phenotype which could account for early recurrence of HCC patients. Loss of Serine/Threonine kinase 11 (STK11) expression was also correlated with increased vascular invasion, shorter disease free survival (DFS) and OS in a Chinese cohort of HCC patients^[14]. Although these oncogenes and tumour suppressors had been correlated with HCC prognosis, their prognostic value remains contradictory. Kondo *et al.*^[15] had shown that C-met overexpression has been linked to an increase in HCC recurrence, while this correlation was not observed in a recent publication by Lee *et al.*^[16]. Although tumour suppressor p16 has been found to be widely inactivated in HCC, p16 has not been shown to be a prognostic marker for HCC^[17,18].

The heterogeneity of HCC suggests that various molecular players in related pathways are likely to be differentially regulated during the process of HCC recurrence. Omics based studies provide the opportunity to provide quantitative information on global expression profiles of various biomolecules in HCC progression in a high throughput and bias-free manner. The identification of dysregulated biomolecules can be used as prognostic biomarkers to predict HCC recurrence. In this review, we surveyed the application of “omics” for identification of prognostic biomarkers for predicting HCC recurrence. Emphasis will be placed on transcriptomics, proteomics and metabolomics, which studies the gene, protein and metabolite expression levels respectively.

TRANSCRIPTOMIC STUDIES AND GENE EXPRESSION PROFILING

Transcriptomics involves the study of expression of all transcribed genes in a genome wide manner. This analysis can be conducted in a high throughput form in the form of DNA microarrays, where cDNA is hybridized onto DNA probes that are present on a chip. Such analysis allows us to profile and identify genes that are differentially expressed with different disease states and clinical outcomes. Among all the global profiling technologies available, DNA microarray and related sequencing techniques are the most commonly used method to obtain molecular “signatures” for prediction of HCC recurrence. Transcriptomic studies that generate information for prediction of recurrence fall into two major groups. The first group of studies uses clinical information to segregate and develop a gene signature that could be used to predict the possible clinical outcome in a separate cohort. The second group of studies identifies distinct subgroup of patients that have the defined phenotype. In the fol-

Table 1 Gene expression studies of hepatocellular carcinoma for prediction of recurrence

Ref.	Sample type	Screening platform	Virus type	Characteristics for distinguishing groups	Number of marker genes	Predictive accuracy for recurrence
Iizuka <i>et al</i> ^[19] (2003)	Tumour tissues	Oligonucleotide microarray	HBV < HCV	Early IHR within 1 yr after surgery	12	25 in 27 independent patients (93%)
Kurokawa <i>et al</i> ^[20] (2004)	Tumour tissues	PCR based array	HBV < HCV	Early IHR within 2 yr after surgery	20	29 in 40 independent patients (72.5%)
Budhu <i>et al</i> ^[35] (2006)	Non-tumour tissues	cDNA microarray	Almost all HBV	Venous invasion or extrahepatic metastasis	17	87 of 95 independent patients (92%)
Ho <i>et al</i> ^[34] (2006)	Tumour tissues	cDNA microarray	HBV > HCV	Venous invasion	14	26 of 35 independent samples (74.3%)
Lee <i>et al</i> ^[37] (2006)	Tumour tissues	Oligonucleotide microarray	HBV > HCV	Hepatoblast gene signature	907	$P < 0.001$ in 66 patients (Probability of recurrence)
Okamoto <i>et al</i> ^[21] (2006)	Non-tumour tissues	cDNA microarray	All HCV	Single nodular HCC vs multicentric HCC	36	30 of 40 training samples (75%)
Wang <i>et al</i> ^[22] (2007)	Tumour tissues and non-tumour tissues	Oligonucleotide microarray	HBV > HCV	HCC recurrence	57	84% in 25 independent samples, sensitivity 86%, specificity 82%
Hoshida <i>et al</i> ^[23] (2008)	FFPE non-tumour tissues	DASL assay	HBV < HCV	Late recurrence more than 2 yr after resection	132	$P = 0.003$ in 224 patients in validation set (Probability of late recurrence)
Somura <i>et al</i> ^[24] (2008)	Tumour tissues	Oligonucleotide microarray/qRT-PCR	HBV < HCV	Early IHR within 1 yr after surgery	3	35 of 43 independent patients (81.4%)
Tanaka <i>et al</i> ^[25] (2008)	Tumour tissues	Oligonucleotide microarray	HBV > HCV	Aggressive recurrence exceeding Milan Criteria	1 (AURKB)	54 of 67 independent patients (80.5%)
Woo <i>et al</i> ^[26] (2008)	Tumour tissues	Oligonucleotide microarray	All HBV	Early IHR within 1 yr after surgery	628	$P = 0.0018$ in 139 independent patients (Probability of early recurrence)
Yoshioka <i>et al</i> ^[27] (2009)	Tumour tissues	Oligonucleotide microarray	HBV < HCV	Early IHR within 2 yr after surgery	172	$P < 0.0001$ in 97 independent patients (Probability of RFS)
Roessler <i>et al</i> ^[28] (2010)	Tumour tissues	Oligonucleotide microarray	HBV > HCV	Early IHR within 2 yr after surgery	161	$P = 0.0057$ for cohort 1, $P = 0.017$ for cohort 2 (Probability of RFS)
Tsuchiya <i>et al</i> ^[29] (2010)	Non-tumour tissues	Oligonucleotide microarray	All HCV	Late recurrence more than 1 yr after resection	38	$P < 0.0001$ in 44 training samples (Probability of RFS)
Woo <i>et al</i> ^[38] (2010)	Tumour tissues	Oligonucleotide microarray	HBV > HCV	Cholangiocarcinoma-like signature	625	$P = 0.037$ in cohort 1 of 61 patients, $P = 0.004$ for cohort 2 of 78 patients (Probability of RFS)
Weng <i>et al</i> ^[30] (2012)	Tumour tissues, PBMC	Oligonucleotide microarray	All HBV	Early IHR within 1 yr after surgery	3	$P < 0.001$ in 80 independent patients (Probability of RFS)
Xieraili <i>et al</i> ^[31] (2012)	Tumour tissues	Oligonucleotide microarray	HBV < HCV	Early IHR	1 (VIL1)	$P = 0.025$ in 90 independent patients (Probability of RFS)
Tsunedomi <i>et al</i> ^[32] (2013)	Tumour tissues	Oligonucleotide microarray	All HCV	Early IHR within 1 yr after surgery	1 (ABCB6)	89% sensitivity, 55% specificity, 86% PPV, 62% NPV in 20 independent patients

Under the heading "virus type", HBV > HCV means that more patients in the study are HBV positive compared to HCV positive patients. HBV < HCV means there are more HCV positive patients in the study. DASL: Complementary cDNA-mediated annealing, selection, extension and ligation; IHR: Intrahepatic recurrence; RFS: Recurrence free survival; PBMC: Peripheral blood mononuclear cells; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma.

Following section, we will review some of the main discoveries that were obtained from the transcriptomic studies (Table 1) and the biological significance behind the gene signatures.

Predictive signatures obtained using recurrence as sample classifier

Most of the gene-profiling studies fall under this category, where early or late recurrence is used as the sample classifier for derivation of gene signatures that can predict recurrence^[19-32]. Studies that are interested in predicting early recurrence commonly used HCC tumour tissues

as molecular changes leading to intrahepatic metastasis seems to be programmed within the primary tumour. Most of the earlier studies had included a heterogeneous study group in both the discovery and validation set to reflect the clinical setting where most patients would have vastly different clinicopathological features. In some studies, homogenous patient cohorts were chosen in the discovery phase to minimize confounding factors that might affect the molecular signatures of tumour samples. These include profiling patients with specific etiology [hepatitis B virus (HBV)-only^[26,30] and hepatitis C virus (HCV)-only^[21,29,32]] and patients with no clinicopathological fac-

tors that were correlated to early recurrence^[27]. Gene profiling experiments had shown an overexpression of genes involved in proliferation^[26,27,30], metastasis^[26, 31] and immune evasion^[19,20]. In particular, SP1 and PPAR α were identified by Woo *et al*^[26] to be the common regulators of many genes overexpressed and downregulated in the high risk group respectively, which makes them attractive targets of drug design for treatment of HCC.

Late recurrence is often associated with the development of multicentric recurrence of HCC. Chronic infection by HBV and HCV results in the production of a cirrhotic liver which would promote the formation of *de novo* tumours, leading to late recurrence. Due to the difference in clonal origins of the recurrent and the primary tumour^[33], non-tumour cirrhotic tissues were used for prediction of late recurrence. In an effort to identify gene signatures related to multicentric recurrence, Okamoto and colleagues profiled the gene expression of non-cancerous liver tissue of 40 HCV positive patients with single nodular and multicentric HCC group^[21]. A 36 gene signature was used to create a prediction score which could predict the risk of patients of multicentric recurrence, with an increase in prediction score conferring a higher risk of multicentric recurrence. However, no validation studies were performed to rate the performance of the prediction score in an independent data set.

Hoshida *et al*^[23] studied the feasibility of gene-expression profiling in formalin-fixed, paraffin-embedded (FFPE) tissues, in which gene expression profiling of fixed tumour and adjacent non-tumour tissues from 105 patients were performed to obtain gene signatures that could predict overall survival and recurrence. Although they were unable to obtain a gene signature that could discriminate between patients with early recurrence from profiling of tumour tissues, they developed a 132 late-recurrence gene set (recurrence after 2 years of surgery). The gene set was validated using FFPE non-tumour tissues from two other countries with different etiology and clinicopathological features, thus suggesting the robustness of the late recurrence signature.

Lastly, to understand gene expression signatures in HCV patients, Tsuchiya and colleagues profiled the tumour and non-tumour tissues of HCV positive patients^[29]. Similar to Hoshida *et al*^[23], a 11 gene set was able to segregate patients with and without recurrence one year after resection. Bioinformatics analysis revealed that hepatic nuclear factor 4-alpha and interferon gamma centred interactomes as the top two significant networks, suggesting the role of chronic HCV infection in promoting late recurrence and disease progression.

Predictive signatures based on phenotype linked to recurrence

Transcriptomics studies in this group define the sample with specific phenotypes that are usually correlated with either recurrence or aggressive nature of tumours. These phenotypes act as surrogate markers for prediction of recurrence in patients. The desired phenotype can be related to pathological features that are correlated with the

predictive parameters, such as vascular invasion and metastasis. Ho and colleagues reported a 14 gene set which could classify patients according to likelihood of vascular invasion^[34]. They had demonstrated the utility of this gene set in predicting recurrence after surgical resection for HCC in an independent cohort of American Joint Committee of Cancer stage I patients without histological evidence of vascular invasion. Budhu *et al*^[35] profiled the noncancerous hepatic tissues from patients with venous or extrahepatic metastasis and patients without metastasis to understand the role of the hepatic microenvironment in promoting metastasis. An immune response signature, corresponding to a global Th1/Th2-cytokine like shift was observed in patients with metastasis. A 17 gene list reflecting this shift in immune response was demonstrated to be able to segregate patients with and without metastasis, and this gene signature was able to predict both recurrence and survival of patients. Although both gene signatures have demonstrated the prognostic ability to predict recurrence, venous invasion might not be present in the tumours of all recurrent patients. Hence, the applicability of these gene sets in predicting recurrence might be limited.

Roessler *et al*^[28] analysed the capability of a previously generated tumour-based metastatic signature in predicting recurrence of patients. The metastatic signature was able to predict early recurrence and overall survival of two independent cohorts with high sensitivity and good specificity. Importantly, the two cohorts have different etiological features, with one cohort consisting of HBV-positive Chinese patients, and the other cohort consisting of a mix of Chinese, European and American patients with different Hepatitis viral infection. This suggests the robustness of the classifier in predicting early recurrence and overall survival in a heterogenous group of patients. Combination of the gene signature with other clinicopathological data such as Barcelona-Clinic Liver Cancer staging and AFP levels improved prediction outcome.

Cancer stem cells have the ability to self-renew, differentiate and grow into a new tumour and are usually refractory to apoptosis. These cells do not die after conventional chemotherapy and often leads to the growth of tumour after curative treatment, resulting in recurrence. These cells are believed to arise from normal stem cells or progenitor cells^[36]. HCC tumours bearing signatures of hepatic progenitor cells might possibly contribute to a poor prognosis, resulting in earlier recurrence and shorter overall survival. Two studies had linked the use of such signatures in predicting recurrence and survival of patients. Lee *et al*^[37] and colleagues integrated data from rat fetal hepatoblasts and adult rat hepatocytes, together with human HCC to identify HCC tumours with similar gene expression as hepatoblasts^[37]. The hepatoblast subgroup (Hb subtype) was independently associated with poorer survival and recurrence. Tumours in the Hb subtype exhibit higher expression of hepatic oval cells as well as genes suggesting the central role of JUN overactivation in recurrence and poor survival of Hb subtype patients.

Cholangiocarcinoma (CC) is one of major type of primary liver cancer with poorer prognosis compared to

HCC. An intermediate form of combined hepatocellular-cholangiocarcinoma (CHC) was suggested to derive from bipotential liver stem cells. To identify cholangiocarcinoma-like traits in HCC which might identify patients with poorer prognosis, Woo *et al.*^[38] performed a genome wide profiling to identify differentially expressed genes between HCC and CC. The CC signature included well known biomarkers for CC and hepatic progenitor cells and HCC patients with CC signature (CLHCC) exhibited a shorter recurrence free survival and overall survival. The CC signature was validated on two independent cohorts of subjects, with tumours having CC signature showing shorter RFS and OS. A combination of CC and stem-cell (ES) signatures were able to further segregate patients with different prognosis, with CC and ES positive patients having the worst prognosis for RFS and OS.

Signatures based on defined phenotypes provide information with regards to the underlying biology behind recurrence through the identification of subgroups of patients with higher risk of recurrence based on specific phenotypes linked to recurrence and aggressive cancer progression. However, the predictive accuracy of such signatures may be lower than signatures based on clinical data since these signatures were used as surrogates for prediction of recurrence.

Gene signatures from HBV and HCV treatment

HBV and HCV infection are the most important etiological factors for HCC development, hence antiviral therapies after resection may be useful as adjuvant therapy to improve prognosis of the patients. The current approved treatment for hepatitis B infection are interferon- α (IFN- α) and nucleos(t)ide analogues (NA), while treatment for hepatitis C infection involves the combination of pegylated IFN- α and guanosine analog ribavirin (RBV). Several studies had compared the prognosis of HCC patients with and without antiviral treatments. In general, these studies had demonstrated the beneficial effects of antiviral treatments in preventing HCC recurrence, especially in patients with HCV infection. These results were summarized in a review article by Du *et al.*^[39].

Transcriptomic studies had been performed on two different types of treatment for HCV infection to understand the molecular mechanism and the possible side effects of treatment. Changes in gene expression of the blood mononuclear cells (PMBC) in patients with chronic HCV infection treated with IFN- α -RBV treatment were assessed using DNA microarray^[40,41]. DNA sensing pathways and RIG-I like receptor signaling pathways were upregulated and ribosomal pathway was downregulated upon IFN- α -RBV treatment. The downregulation of ribosomal pathway suggests a suppressive effect of IFN- α /RBV treatment in protein translation, which could explain the side effects of interferon treatment (such as fatigue) as well as its inhibitory effect on viral replication. The other study by Honda *et al.*^[42] involved the understanding of molecular effects of Peretinoin against recurrent HCC. Peretinoin is an acyclic retinoid which was previously reported to be efficacious in reducing the

incidence of recurrent HCC. Gene expression profiling of liver tissues of HCV-positive HCC patients before and after peretinoin treatment showed that the expression levels of retinoid target genes, interferon target genes and tumour suppressor related genes were markedly elevated after peretinoin treatment. This was accompanied by a decreased expression of Wnt, Mammalian target of rapamycin and tumour progression related genes. This study highlighted the molecular basis for understanding the efficacy of peretinoin in preventing HCC recurrence, as well as the major molecular pathways that could be related to HCC recurrence.

Limitations of transcriptomics

Many gene profiling experiments had been performed in the search for a gene signature that could predict HCC recurrence with high accuracy. The predictive accuracy of these studies are promising, however these studies usually use a small sample size which might artificially inflate the predictive accuracy of the gene signature. There are few genes that overlap between these studies, which might be due to the use of different microarray platform, sample population, definition of the sample labels (*e.g.*, different cut-off times used to define early recurrence) and the type of tissues profiled (frozen tissues *vs* FFPE). Villanueva *et al.*^[43] recently tested the predictive value of 22 published gene signatures that were reported to be prognostic for HCC on FFPE tissues corresponding to 201 patients. Only two signatures, namely G3 signature from tumour tissues^[44] and poor survival signature from non-tumour tissues^[23] were associated with shorter RFS and OS, suggesting the validity of the signature depends on the criteria set for the sample labels and the type of tissue profiled (frozen tissues *vs* FFPE tissues). In an effort to demonstrate the robustness of the gene signatures, cross-platform validation of signatures on patient cohorts with different etiology had been performed in several studies. However, there are still no gene signatures that had shown clinical significance in predicting recurrence on large sample sizes. A meta-analysis on the existing gene expression studies would provide the impetus to select and determine a gene signature that might best predict recurrence in patients, which could potentially be used clinically. In addition, identification of pathways that are commonly found to be dysregulated in different gene signatures might grant more insights to biological changes related to HCC recurrence. This is because gene profiling experiments are inherently noisy and some of the genes in the gene signature might not discriminate the recurrence and non-recurrence groups.

MIRNA AND HCC RECURRENCE

MicroRNAs (miRNA) are highly conserved, endogenous single-stranded non-coding RNA of 17-25 nucleotides. Long precursor RNAs with stem-loop structures are processed by Drosha and DICER to form mature miRNAs. Mature miRNAs post-transcriptionally regulate gene expression *via* base pairing with the 3'-UTR of target

Table 2 miRNA for prediction of recurrence in hepatocellular carcinoma

Ref.	Sample type	Screening platform	Virus type	Characteristics for distinguishing groups	Number of candidate miRNA	Predictive accuracy for recurrence
Fornari <i>et al</i> ^[53] (2009)	Tumour and non-tumour tissues	qRT-PCR	HBV < HCV	miR-122 levels	1 (miR-122)	$P = 0.05$ for 45 independent patients (Recurrence rate)
Fornari <i>et al</i> ^[54] (2010)	Tumour and non-tumour tissues	qRT-PCR	HBV < HCV	Late recurrence beyond two years after surgery	1 (miR-199a-3p)	$P = 0.043$ for 36 independent patients (Recurrence rate)
Augello <i>et al</i> ^[47] (2012)	FFPE tumour tissues	qRT-PCR	All HCV	Stages of HCC progression	18	$P = 0.042$ for 61 independent patients (Percent Recurrence)
Han <i>et al</i> ^[52] (2012)	FFPE tumour tissues	qRT-PCR	Mostly HBV	Recurrence after OLT	1 (miR-155)	$P < 0.001$ for 100 training patients (RFS)
Huang <i>et al</i> ^[51] (2012)	Non-tumour tissues	qRT-PCR	HBV > HCV	Early IHR within 6 mo after surgery	6	$P < 0.001$ for 216 independent patients
Shih <i>et al</i> ^[48] (2012)	Tumour and non-tumour tissues	qRT-PCR	Not mentioned	HCC and non-tumour	15	$P = 0.005$ for 68 training samples and 13 independent samples (RFS)
Xia <i>et al</i> ^[50] (2012)	Tumour and non-tumour tissues	qRT-PCR	Mostly HBV	early IHR within 2 years after surgery	1 (miR-214)	$P = 0.009$ for 50 independent patients (RFS)
Zhu <i>et al</i> ^[49] (2012)	FFPE tumour and non-tumour tissues	qRT-PCR	All HBV	Early IHR after surgery	1 (miR-29a-5p)	AUC = 0.708 for 112 independent patients

OLT: Orthotopic liver transplantation; IHR: Intrahepatic recurrence; RFS: Recurrence free survival; AUC: Area under curve; HCV: Hepatitis C virus; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; qRT-PCR: Quantitative reverse transcription polymerase chain reaction.

mRNAs, resulting in cleavage or translational repression. There are more than 2000 mature miRNAs found in human (miRBase, Release 20.0) (<http://www.mirbase.org/>)^[45], and they are involved in several biological events at physiological and pathological conditions, such as embryonic development, cell growth, differentiation, apoptosis, and invasion^[46].

In the following section, we discuss some of the recent studies on large scale profiling of miRNAs in the discovery of biomarkers for HCC recurrence (summarized in Table 2). The postoperative recurrence rate and OS in the HCC patients could be used to determine their values as prognostic marker(s), and as potential target in anti-HCC therapies. In some of these studies, the miRNA biomarkers were also found to be associated with hepatocarcinogenesis, orthotopic liver transplantation (OLT), or chemotherapy drug treatment for HCC.

miRNA as molecular classifiers for tumour progression

A low-density array was used to profile the levels of 664 miRNAs in 60 hepatitis C-positive liver tissues from patients of different stages of hepatocarcinogenesis to identify miRNAs that are deregulated during HCV-related liver carcinogenesis^[47]. A 18-miRNA signature was found to distinguish cirrhosis, liver dysplasia and HCC lesions. Among these miRNAs, miR-515-3p, miR-518a-3p, miR520f and miR-525-3p were members of the chromosome 19 miRNA cluster (C19MC), and were selectively over-expressed in HCC. miR-512-3p was significantly over-expressed in early HCC as compared with high-grade dysplasia, whereas miR-519d and 525-3p were over-expressed in tumour as compared with either low- or high-grade dysplastic nodules in the HBV-related samples. In another 61 HCC and matched cirrhosis cases, 11 miRNA members of C19MC were up-regulated in HCC compared to non-neoplastic counterparts. The increase in C19MC miRNA was also associated with poor clinico-

pathological features, tumour recurrence and shorter OS time. This was the first study reporting the over-expression of chr19 miRNA in predicting HCC recurrence and OS, suggesting C19MC as potential target for therapies strategies against HCC.

Expression profiling of miRNAs in 68 HCC and 21 non-tumoural liver tissues has identified 15 miRNAs as potential classifier of HCC^[48]. Among these miRNAs, miR-214 was found to be down-regulated in HCC, and this was associated with short RFS and OS. The deregulation of miR-214 was also associated with higher serum AFP, and advanced tumour stage in HCC patients. Furthermore, over-expression of miR-214 in HCC cells inhibited xenograft tumour cell proliferation, microvasculature of tumours and surrounding tissue, and suppressed orthotopic xenograft tumour formation in nude mice. miR-214 was thus proposed as a tumour suppressor of HCC associated with poor prognosis, and implicated in dedifferentiation, induction of stemness, and tumour transformation of hepatocytes. Lastly, the authors showed that the miR-214/HIDGF paracrine pathway might be responsible for regulation of angiogenesis during HCC development, suggesting this pathway as potential target for anti-HCC therapy since angiogenesis plays a crucial role in HCC development.

miRNA as predictive signatures of recurrence

In a recent report, Zhu *et al*^[49] analysed the miRNAs in microdissected HBV-related HCC tissues to detect miRNAs that can predict early recurrence after HCC resection. Increase in miR-29a-5p was found to be associated with early tumour recurrence and shorter OS, including patients with early stage HCC. The authors showed that miR-29a-5p was an independent prognostic indicator for early tumour recurrence after HCC resection. This association of high miR-29a-5p with metastasis-related early recurrence of HCC concurs with deregulation of miR-29

family members in cancers. However, further functional studies would be necessary to understand the mechanism of miR-29a-5p's role in HCC recurrence to aid the development of cancer therapeutics.

In a study by Xia *et al.*^[50], down-regulation of miR-NA-214 was also found to be associated with early recurrence of HCC, cancer cell invasion, and stem-like traits. The authors also demonstrated that the degree in the reduction of miR-214 correlated with the invasiveness and metastatic ability of liver cancer cell lines. Moreover, overexpression of miR-214 inhibits growth and invasion *in vitro* and tumourigenicity *in vivo*. This study also found that enhancer of zeste homologue 2 (EZH2) and β -catenin (CTNNB1) are down-stream targets of tumour suppressor miR-214. The increase in EZH2 and CTNNB1, and the decrease in miR-214 in HCC patients correlated with early recurrence and could predict poor survival. The results derived from this study could help in the development of therapeutics against HCC.

Huang *et al.*^[51] has compared the miRNA profiles of HCC liver tissues with that of non-tumour liver tissues to discover postoperative prognostic predictors for HCC. A panel of 20 miRNAs was identified from this study of 12 HCC patients (6 with better prognosis and 6 with poorer prognosis), of which high expression of miR-155, miR-15a, miR-432, miR-486-3p, miR-15b, and miR-30b were significantly associated with shorter RFS. miRNA-155 were also found higher in tumour tissues of patients with post-OLT HCC recurrence as compared to those of non-recurrence patients in an independent study^[52]. Patients with higher miR-155 expression had poorer recurrence-free survival and shorter OS, independent of serum AFP level. Functional studies on HCC cell lines implicate the role of miR-155 in proliferation, survival and invasion^[51,52]. Thus, miR-144 could be a good prognostic marker for recurrence risk stratification and target for anticancer therapy in HCC patients.

The reduction of miR-122 level, which is common in HCCs, affects HCC development and progression, and low miR-122 expression was shown to correlate with a shorter time to recurrence in patients resected for HCC^[53]. miR-122 was found to promote the stability and transcriptional activity of p53, and lowers the invasiveness of HCC-derived cell lines, partially through regulating cyclin G1. Furthermore, miR-122, and silencing of cyclin G1 increases the sensitivity of the cancer cells to doxorubicin, one of the commonly used drugs for intermediate-advanced HCCs. In another study by Fornari *et al.*^[54], a shorter time to recurrence after HCC resection was found in patients with lower miR-199a-3p levels, and re-expression of miR-199a-3p in HCC-derived cell lines resulted in blockage of G₁-S transition, lowered invasion capability, and increased response to doxorubicin treatment. These studies suggested that combined chemo- and miRNA-based therapy is a potential strategy for HCC treatment. Although miRNAs are potential clinically useful biologic metrics to guide patient selection and therapeutic interventions, it should be noted that there could be issues with the toxicity and off-target effects for

miRNA as therapies in HCC.

PROTEOMICS STUDIES OF HCC

Proteomics is another key area of study which has been extensively used in cancer studies. The dynamic nature of proteins and its response to external and internal stimuli in the disease state might not be apparent from the transcripts, making proteomic studies an attractive method to understand disease progression. Quantitative expression proteomics allow for identification of proteins that are differentially regulated between two groups of patients, which could be used to identify prognostic biomarkers for HCC recurrence. Such studies involve the separation and identification of the proteins in a complex mixture such as tissue lysates. Separation of proteins is commonly performed using two-dimensional electrophoresis (2DE), which separates proteins according to their pI and molecular weight on a polyacrylamide gel, while separation of peptides is usually performed using liquid chromatography. This is coupled to mass spectrometry such as matrix assisted laser desorption/ionization or electrospray ionization for identification of proteins at a high level of automation and sensitivity. Limited proteomic studies have been performed to identify prognostic biomarkers that could predict recurrence in HCC patients after liver resection or liver transplantation. In the following section, we will discuss about the main findings from these papers (Table 3).

Proteomics identification of predictive biomarkers for recurrence

Yokoo *et al.*^[55] used a two-dimensional difference gel electrophoresis (2D-DIGE) approach to compare the proteome of 12 patients with early intrahepatic metastasis within 6 mo after surgery with 15 patients who did not have recurrence within 2 years after resection. A total of 23 differentially expressed proteins were identified and these proteins were involved in wide range of biological processes. This 23 protein list was validated on a set of 13 patients with a high predictive accuracy of 92%. However, the validation method was performed using 2D-DIGE, which required skilled personnel to perform and may not be easily translated.

In a similar study, Yi *et al.*^[56] used a differential proteomic approach with 2DE to profile the tumour and non-cancerous tissue of 103 Chinese HBV-positive patients. Mortalin was found to be overexpressed in the tumours of patients who recurred within 1 year post surgery, with high predictive accuracy and sensitivity. Elevated mortalin levels were correlated with the presence of venous invasion and advanced tumour stages, while increased mortalin levels were found in HCC cell lines with higher metastatic ability. The authors proposed that mortalin played a role in promoting the metastatic property of HCC and is a positive predictor for early HCC recurrence.

A similar approach was used by Cheng and colleagues to identify protein biomarkers that could discriminate patients with higher risk of recurrence after liver trans-

Table 3 Proteomic studies of hepatocellular carcinoma for prediction of recurrence

Ref.	Sample type	Screening platform	Characteristics for distinguishing groups	Candidate biomarkers	Validation method
Yokoo <i>et al.</i> ^[55] (2007)	Tumour tissues	2D-DIGE, MALDI-TOF MS	Early IHR within 6 mo after resection	23 protein panel	2D-DIGE
Orimo <i>et al.</i> ^[59] (2008)	Tumour and non-tumour tissues	2D-DIGE, LC-MS/MS	Histological differentiation of tumours	Adenomatous polyposis coli-end-binding protein 1 (EB1)	IHC
Yi <i>et al.</i> ^[56] (2008)	Paired tumour and non-tumour tissues	2DE, MALDI-TOF/TOF MS	Early IHR within 1 yr after resection	Mortalin (HSPA9)	qPCR, Immunoblotting, IHC
Bai <i>et al.</i> ^[58] (2009)	Tumour tissues	cICAT, 2DLC-MS/MS	Recurrence after liver transplantation	Calpain small subunit 1 (CAPN4)	qRT-PCR, Immunoblotting, TMA
Cheng <i>et al.</i> ^[57] (2011)	Tumour tissues	2DE, MALDI-TOF/TOF MS	Recurrence after liver transplantation	N-myc downstream-regulated gene 1 (NDRG1)	Immunoblotting, IHC
Kanamori <i>et al.</i> ^[60] (2011)	Tumour and non-tumour tissues	2DLC-MS/MS	Tumour and non-tumour	Talin-1 (TLN1)	IHC

IHR: Intrahepatic recurrence; IHC: Immunohistochemistry; TMA: Tissue microarray; qRT-PCR: Quantitative reverse transcription polymerase chain reaction; 2D-DIGE: Two-dimensional difference gel electrophoresis; MALDI: Matrix-assisted laser desorption/ionization; LC-MS: Liquid chromatography-mass spectrometry.

plantation^[57]. 2DE profiling of tumour tissues was performed on 143 patients with and without recurrence after liver transplantation. N-myc Downstream-regulated gene 1 (NDRG1) was significantly overexpressed in patients with recurrence compared to non-recurrence patients. Knockdown of NDRG1 in HepG2 cells reduced growth, invasion and migration ability of the cells, thus suggesting the role of NDRG1 in promoting proliferation and metastasis. Positive NDRG1 expression was also correlated with presence of venous invasion, advanced tumour staging, high serum AFP concentration and recurrence.

Likewise in the search of biomarkers for HCC recurrence after liver transplantation, Bai and colleagues utilized the cleavable isotope-coded affinity tag technology, and 2-Dimensional Liquid Chromatography coupled with tandem mass spectrometry (2D-LC MS/MS) to quantitate relative protein changes between patients with or without recurrence after liver transplantation^[58]. A total of 52 differentially regulated proteins was identified, among which calpain small subunit 1 (Capn4) was demonstrated to be consistently overexpressed in both tumour tissues of recurrent patients and HCC cell lines with higher metastatic ability. Knockdown of Capn4 was shown to be an independent prognostic factor for recurrence and survival of HCC patients through multivariate analysis.

Proteomics identification of biomarkers related to HCC progression and its correlation with recurrence

Histological differentiation has been one of the pathological factors for poor prognosis. To identify molecular players in HCC differentiation, Orimo *et al.*^[59] profiled a total of 27 HCC tissues with different degrees of histological differentiation using 2D-DIGE. 26 unique proteins were identified to be differentially expressed, of which Adenomatous Polypsis Coli-End Binding Protein 1 (EB1), which is controlled by factors linked

to HCC malignancy such as c-Myc, RhoA and CDC42, was chosen for further validation. Increased EB1 expression was found to be associated with poorer histological differentiation, higher cumulative recurrence rate and shorter overall survival.

In another study to identify proteins related to HCC progression, Kanamori and colleagues used 2D-LC MS/MS to interrogate the proteome of 4 early HCC and 4 non-HCC tissues derived from two cases of liver transplant surgery^[60]. Among the 83 proteins that were differentially expressed in the tumour and non-tumour tissues, Talin-1, a cytoskeletal protein was chosen for further validation. Immunohistochemical analysis revealed the direct correlation of Talin-1 expression with poor histological differentiation, and using 50% cells staining as a cutoff, patients with more than 50% cell staining were shown to have significantly shorter disease free survival rate.

Limitations of proteomics

In proteomics studies, validation of results obtained from the discovery phase would usually be performed using antibody based methods, such as immunoblotting or immunohistochemical analysis, which are costly. Hence, proteomic studies are often followed by validation of single potential prognostic markers that usually has functional implications related to HCC progression/metastasis or has the highest fold difference between the sample groups. However, due to the highly heterogeneous nature of HCC recurrence, a single prognostic marker might not be specific and sensitive enough to detect patients with high risk of recurrence. Multiple prognostic markers could possibly provide higher predictive accuracy as compared to single prognostic marker.

METABOLOMICS STUDIES OF HCC

Metabolomics is a large scale quantitative and qualitative

Table 4 Advantages and limitations of the three different omics methodologies

Method	Advantages	Limitations
Transcriptomics	Large dataset of dysregulated genes identified, provides insights to biological mechanisms of disease Affordable price Has provided successful example of translation to clinical use (<i>e.g.</i> , Oncotype Dx™ in predicting breast cancer recurrence)	Differences in platform contribute to lack of overlap in signatures between different studies High possibility of noise present in the gene list Poor correlation between transcript and protein levels
Proteomics	Direct measurement of biological effectors Validation method (IHC and TMA) routinely performed in pathology labs	Small number of validated targets Price, availability and quality of antibodies for validation work
Metabolomics	Amenable to different types of samples which can be obtained in a non-invasive manner	Lack of large-scale validation of metabolite signatures Limited biological information obtained

IHC: Immunohistochemistry; TMA: Tissue Microarray.

study of small molecules (< 1 kDa), which commonly involves the use of high-throughput liquid chromatography (*e.g.*, high performance liquid chromatography or ultra performance liquid chromatography) and mass spectrometry or nuclear magnetic resonance spectroscopy for an unbiased comparison of global metabolite profiles in biological samples, such as urine, serum and tissues. Importantly, alterations in the metabolites represent downstream events of transcriptome and proteome changes, and reflect dynamic responses to endogenous and exogenous factors, such as pathological, environmental or lifestyle effects. Metabolomics study of cancers was largely based on the hypothesis by Otto Warburg: tumour cells contain altered metabolic profiles mainly due to modified mitochondrial respiration and glycolytic pathways. Current findings from metabolomics have concurred with the Warburg phenomenon of altered tumour metabolism, with an increased glycolysis but reduced mitochondrial oxidative phosphorylation. Being one of the largest metabolic organs in the body, HCC progression would result in huge alterations in the metabolome of liver, making metabolomics an attractive field for identifying diagnostic and prognostic biomarkers for HCC. Metabolomics has been applied to look for diagnostic biomarkers^[61-67]. To the best of our knowledge, there was only one study that applied metabolomics in search for predictive biomarkers for HCC recurrence, which would be discussed below.

In a pioneer urine metabolomics study to identify differential metabolic changes in patients with early HCC recurrence after resection, Ye *et al.*^[68] used GC-TOF/MS to profile urine samples of patients before and after surgery. Energy metabolism, amino acid metabolism, nucleoside metabolism, tricarboxylic acid cycle, and gut flora metabolism were found altered after surgical removal of tumour. This work identified metabolic signatures of HCC recurrence comprising of up-regulated lactate excretion, succinate production, purine and pyrimidine nucleosides turnover, glycine, serine and threonine metabolism, aromatic amino acid turnover, cysteine and methionine metabolism, and glyoxylate metabolism. These findings reflect the increase in proliferation, decrease in mitochondria oxidative phosphorylation and increased oxidative stress in recurrent patients that is correlated

with the growth and increase in tumour load. In addition, 5 metabolites, namely ethanolamine, lactic acid, acotinic acid, phenylalanine and ribose were determined to be combinatorial biomarkers for discriminating early recurrent patients from non-recurrent patients post-surgery. Findings from metabolomics studies of urine would allow widespread non-invasive screening and surveillance instead of diagnostic tests using AFP and liver ultrasound that are prohibitively expensive. However, validation on a large cohort is needed to confirm the robustness and accuracy of this metabolomics signature in predicting HCC recurrence.

Limitations of metabolomics

Metabolomics is a powerful tool that can be used to quantitatively measure the differences in metabolite abundance between two different disease states. The ability to perform such studies in a large range of biological samples, including urine samples in which collection is easy and non-invasive, makes it an attractive platform for translation to clinical use. However, current studies lack validation of results in large sample cohorts. Moreover, the amount of biological information that could be extracted from metabolomics studies might be limited. This is because metabolic pathways are greatly interlinked and various proteins might be involved in the catabolism/anabolism of a particular metabolite. Hence, it might be difficult to attribute the changes in metabolite abundance to the expression/activity of a particular protein or a pathway.

PERSPECTIVES

HCC is a lethal disease with complex etiology. Despite improvements in detection methods, the prognosis of HCC remains poor, mainly due to the high recurrence rate after surgical interventions. Hence there is a pressing need for prognostic biomarkers that could stratify patients according to risk of cancer recurrence. Understanding the molecular mechanisms behind recurrence could allow for the development of personalized therapy to target recurrence with different biological characteristics. Through the use of omics based methods in studying HCC recurrence, we had generated huge amounts of data on the possible signatures of recurrence. However,

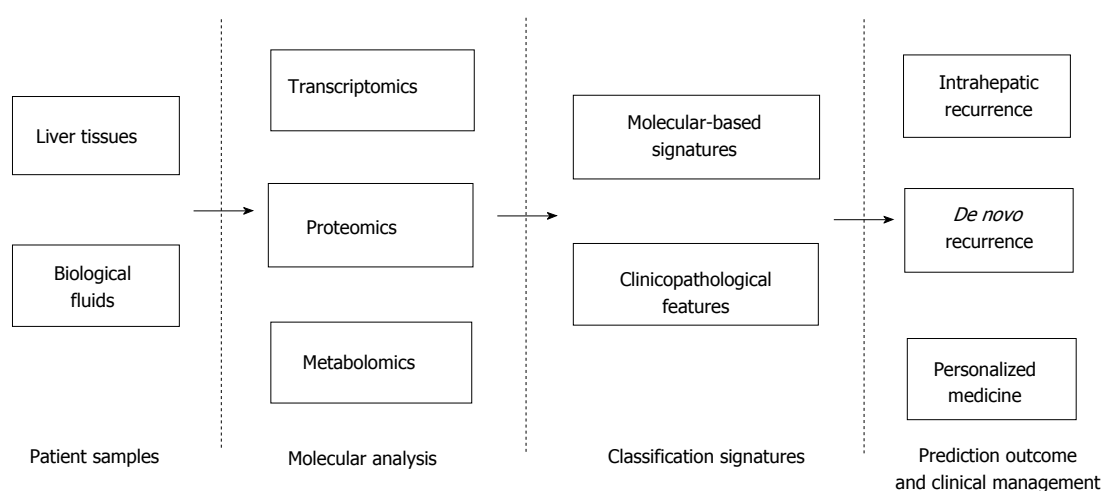


Figure 1 Predictive systems for outcome of hepatocellular carcinoma patients. Tissue samples (tumour and non-tumour) as well as biological fluids (blood, urine *etc.*) are used for molecular analysis *via* omics based methodologies to obtain molecular based signatures for HCC recurrence. The combination of these signatures and clinicopathological features (e.g., HBV or HCV infection, serum AFP levels, tumour staging) would be used to generate predictive systems for intrahepatic recurrence and *de novo* recurrence. Personalised treatment could be generated and administered based on the molecular basis of recurrence. HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus; HCV: Hepatitis C virus; AFP: Alpha-fetoprotein.

such information has not been proven to be useful clinically for HCC prognosis. Systematic evaluation of these data on a large scale in a prospective study would be needed to verify the prognostic accuracy in a statistically convincing manner.

Currently, the molecular mechanisms underlying HCC recurrence remain largely enigmatic. There are no candidate genes that are commonly identified by the three omics methods. Different omics platforms could provide biological insights of HCC recurrence in a complementary fashion, despite their advantages and limitations (Table 4). Integration of data from different omics platform could reinforce the understanding and classification of HCC patients by highlighting common pathways that are dysregulated in subsets of patients (Figure 1). Such studies had already been applied in the metabolomics study, where different metabolomics signatures were associated with different subclasses of HCC patients classified *via* gene expression profiling^[69]. Classification of patients would provide an avenue for personalised medicines which specifically targets the main players leading to recurrence, resulting in better clinical outcomes and improvements in quality of life. With advancements in technology and bio-informatics, the complete characterization of HCC recurrence should be achievable in the near future.

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WJG 20th Anniversary Special Issues (1): Hepatocellular carcinoma

DCE-MRI in hepatocellular carcinoma-clinical and therapeutic image biomarker

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Abstract

Dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) enables tumor vascular physiology to be assessed. Within the tumor tissue, contrast agents (gadolinium chelates) extravasate from intravascular into the extravascular extracellular space (EES), which results in a signal increase on T1-weighted MRI. The rate of contrast agents extravasation to EES in the tumor tissue is determined by vessel leakiness and blood flow. Thus, the signal measured on DCE-MRI represents a combination of permeability and perfusion. The semi-quantitative analysis is based on the calculation of heuristic parameters that can be extracted from signal intensity-time curves. These enhancing curves can also be deconvoluted by mathematical modeling to extract quantitative parameters that may reflect tumor perfusion, vascular volume, vessel permeability and angiogenesis. Because hepatocellular carcinoma (HCC) is a hypervascular tumor, many emerging therapies focused on the inhibition of angiogenesis. DCE-MRI combined with a pharmacokinetic model allows us to produce highly reproducible and

reliable parametric maps of quantitative parameters in HCC. Successful therapies change quantitative parameters of DCE-MRI, which may be used as early indicators of tumor response to anti-angiogenesis agents that modulate tumor vasculature. In the setting of clinical trials, DCE-MRI may provide relevant clinical information on the pharmacodynamic and biologic effects of novel drugs, monitor treatment response and predict survival outcome in HCC patients.

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Key words: Dynamic contrast-enhanced magnetic resonance imaging; Perfusion magnetic resonance imaging; Hepatocellular carcinoma; Angiogenesis inhibitors; Clinical trials

Core tip: Dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) enables tumor vascular physiology to be assessed. Within the tumor tissue, contrast agents extravasate from intravascular into the extravascular extracellular space, which results in a signal increase on T1-weighted MRI. These signal intensity-time curves can be deconvoluted by mathematical modeling to extract parameters that may reflect tumor angiogenesis. DCE-MRI allows us to produce highly reproducible parametric maps of quantitative parameters in hepatocellular carcinoma (HCC). In the setting of clinical trials, DCE-MRI may provide relevant clinical information of novel drugs, monitor treatment response and predict survival outcome in HCC patients.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common tumor and represents the third leading cause of cancer death worldwide^[1]. It is the major cause of death in the cirrhotic patients, beside complications from portal hypertension^[2]. The hypervascular nature and vascular in-out flow pattern of this tumor help differentiation of HCC from other tumors by non-invasive diagnostic criteria, without the necessity of tissue proof^[3]. Like other malignant tumors, previous researches have reported the importance of angiogenesis with the development of HCC^[4-8]. For example, Yamaguchi *et al*^[9] found that vascular endothelial growth factor (VEGF) expression in HCC tissues may be related to the histological grade. Thus, various angiogenesis inhibitors have been developed to treat HCC. Among them, the multikinase inhibitor sorafenib was first approved and validated by two separate phase III trials conducted in Western and Asian countries, respectively^[10,11]. Up to December 2013, there are at least 20 active phase III trials evaluating systemic treatments for advanced HCC (from clinicaltrials.gov-last visit 20th December 2013), with most of studies using sorafenib as combination therapy.

Dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) have been used widely as biomarkers in many early phase clinical trials to evaluate the effects of anti-angiogenic drugs that modulate tumor vasculature^[12-16], and to help effective drug selection and optimal drug dose decision^[17]. For phase III clinical trials, DCE-MRI can serve as a surrogate biomarker to evaluate drug efficacy before the volumetric change of the tumor^[18], and may be associated with progression-free survival and/or overall survival in these patients^[19,20]. The enhancement patterns in HCC obtained by DCE-MRI are influenced by tumor angiogenesis and correlated with tumor microvessel density and VEGF expression^[21]. Thus, suppression of tumor vascular permeability induced by anti-angiogenic agents can be reliably detected and quantified by DCE-MRI. Besides assessing anti-angiogenic agents, DCE-MRI can also be used in the evaluation of response of HCC after other treatments, including transarterial chemoembolization^[22] and radiotherapy^[23]. This review will attempt to summarize the current clinical application of DCE-MRI for HCC patients.

BASIS OF DCE-MRI

DCE-MRI images are obtained by injecting low-molecular-weight gadolinium chelated contrast agent into a vein with a constant rate^[24]. The contrast agent is carried by blood flow into the tissue, causing increased signal intensity (SI) of the T1-weighted images due to the shortening of the longitudinal relaxation time of the tissue^[25]. Within the tissue, the contrast agent passes from the arteries to the capillaries, and then extravasates to the extravascular extracellular space (EES). The rate of contrast agent extravasation to EES in the tumor tissue is determined by vessel leakiness and blood flow. Thus, the signal measured on DCE-MRI represents a combination of perme-

ability and perfusion. DCE-MRI is sensitive to alterations in vascular permeability, extracellular space, and blood flow. To ideally record the signal change in the supplying blood vessel and within the tumor, a fast injection rate of the contrast agent captured with high temporal resolution is required^[26,27].

This signal enhancement of liver perfusion can be quantified either with a semi-quantitative or quantitative analysis. The semi-quantitative analysis is based on the calculation of heuristic parameters that can be extracted from SI curves. In contrast, the quantitative analysis needs computational-based curve fitting algorithms using a bi-compartmental model with arterial input function. The parameters from both analysis methods have been shown to present correlation with tumoral angiogenesis^[28].

SEMI-QUANTITATIVE ANALYSIS

Regarding the semi-quantitative analysis, different parameters that characterize the shape of the normalized SI-time curve can be extracted: (1) area under curve (AUC): expresses the amount of enhancement over a defined period of time (usually from starting increment of the SI-time curve to 60 or 90 s); (2) maximum of SI or Peak enhancement ratio ($(SI_{\text{maximum}} - SI_{\text{baseline}}) / SI_{\text{baseline}}$) of the enhancing curve; (3) wash-in Slope: determines the velocity of enhancement. It is calculated as the maximum change in enhancement per unit time, usually from 20% to 80% range of the increment curve; and (4) mean transit time (MTT): represents the mean time for blood to perfuse a region of tissue and is affected by the blood volume and blood flow in the region under analysis.

The semi-quantitative analysis is widely used because it is easy to calculate without the need of modeling. However, these heuristic parameters are highly affected by the gain factor of the acquisition systems, contrast media volume and injection rate, because the true concentration of contrast agent in the tissues is not estimated. Thus, differences in temporal resolution and injection rates can easily change the shape of SI curves, making comparison and quantification difficult^[26,29]. Moreover, these descriptive parameters provide no physiologic insight into the behavior of the tumor vessels.

QUANTITATIVE ANALYSIS

On the other hand, the quantitative analysis is based on modeling the concentration change of the contrast agent using pharmacokinetic modeling techniques^[30]. An initial conversion step of SI to concentration values is needed. Concentration vs time curves are then fitted using a bi-compartmental model (vessels and EES) with two vascular inputs (aorta and portal vein). The following parameters can be derived from a mathematical model^[26,31]: (1) K^{trans} (forward volume transfer constant): determines the flux of the contrast agent from the intravascular space to the EES. It predominantly represents the vascular permeability in a permeability-limited (high flow) situation, but represents the blood flow into the tissue in a flow-limited

(high permeability) situation; (2) K_{ep} (reverse reflux rate constant): expresses the return process of the contrast agent from the EES to the intravascular space; and (3) V_e (volume fraction of EES): an indirect measure representing the cellular density of the tissue.

These parameters require additional calculations to generate parametric maps obtained after a pixel-by-pixel curve fitting process of the region under analysis. Thus, they are more computationally technical to obtain than the semi-quantitative ones. After generating parametric maps, the mean or median values within region of interests are usually calculated to represent tumor microvasculature, but histogram analysis^[32] or heterogeneity in parametric maps^[33-35] may also provide additional information. For optimum parameter quantification, a high temporal resolution is required to record initial rapid uprising of the SI curve immediately after the contrast agent administration^[36]. The accuracy of these parameters is influenced by curve fitting algorithms^[37,38] and magnitude of motion artifacts^[39].

MODEL SELECTION

Kety^[40] first described the flow-limited tracer uptake in tissue, and since then several pharmacokinetic models have been proposed by Tofts *et al.*^[41], Brix *et al.*^[42] and Larsson *et al.*^[43]. All these models used single source of arterial input function. Because HCC receives major blood supply from hepatic arteries, a single-input two compartment model is commonly used in most articles. However, for liver parenchymal disease or metastatic hepatic tumors which are supplied by both hepatic arteries and portal veins, a dual-input one compartment model by Materne *et al.*^[44] is often used to obtain parameters including arterial blood flow, portal blood flow, hepatic arterial fraction, distribution volume and MTT. For example, several articles used DCE-MRI with Materne model to stage liver fibrosis^[45,46]. Liver perfusion assessed by DCE-MRI revealed increased hepatic arterial fraction and distribution volume with increasing liver fibrosis^[45,47,48].

Recently, a hepatocyte-specific contrast agent was developed and showed different characteristics from traditional gadolinium-based contrast agents. A new model was developed for analysis of hepatic uptake by DCE-MRI using this hepatocyte-specific contrast agent^[49]. Depending on the mathematical model applied and physiological assumptions made, variants of such quantitative parameters are obtained. Hence, when applying tracer kinetic modeling to clinical studies, it is important to state the choice of kinetic model employed at the outset. Currently, there is no consensus as to which kinetic model is best suited to evaluate the liver and HCC, and the development of an international consensus is necessary to allow a wider use of this technique.

Different field strengths employed in the dynamic acquisitions for developing DCE-MRI analysis have been shown to have a direct effect on the results of the pharmacokinetic parameters^[50,51]. The choice of contrast agent molecular properties^[52] and the temporal resolution

of the acquisition have a clear influence on the parameters. To standardize calculations, the acquisition should have enough temporal resolution (less than 2-5 s each image set, during at least 5 min), and voxel-wise statistical analysis is suggested.

CLINICAL APPLICATION OF DCE-MRI

DCE-MRI is helpful to differentiate HCC from colorectal metastasis^[53]. The values of arterial, portal and total blood flow, and distribution volume were significantly higher in the HCC than in the metastatic group, whereas MTT was significantly higher in the metastatic group.

Miyazaki *et al.*^[54] demonstrated that a lower pretreatment distribution volume and high arterial flow fraction was associated with a better response to treatment in patients with neuroendocrine liver metastases treated using yttrium-90 (Y-90)-labeled octreotide (⁹⁰Y-DOTATOC).

DCE-MRI is emerging as a promising method for monitoring tumor response to treatment in HCC patients, and could be used an early imaging biomarker to predict survival outcome of patients. The data are summarized in Table 1.

Wang *et al.*^[55] evaluated thalidomide efficacy in seven patients with advanced unresectable HCC that had failed to respond to prior local therapy. When comparing the MRI parameters for the tumors before and during treatment, they found a statistically significant difference for the peak enhancement, the maximal enhancement, and the enhancement slope percentage between two groups of patients (four had progressive disease, three had stable disease/partial response) with different clinical outcomes.

Liang *et al.*^[23] investigated the changes of the hepatic parenchyma and tumors by DCE-MRI in 19 patients with advanced HCC who received radiotherapy for 50 Gy in 25 fractions. An increased slope and peak of the tumor at week 2 was associated with an improved local response ($P < 0.05$) (Figures 1 and 2). In the parenchyma, an increased slope at week 2 was associated with recurrence outside the radiation fields or with progression over distant sites ($P < 0.05$). These findings emphasized the value of DCE-MRI in the second week after the start of radiotherapy in predicting local tumoral responses or systemic metastasis of HCC after radiotherapy.

Zhu *et al.*^[56] conducted a phase II study of sunitinib, an anti-VEFG receptor tyrosine kinase inhibitor, in 34 patients with advanced HCC. They found significant decreases in K^{trans} and K_{ep} after treatment ($P < 0.0001$). The extent of decrease in K^{trans} was substantially higher in patients who experienced partial response or stable disease compared with that in patients with progressive disease or who died during the first two cycles of therapy. They concluded that rapid changes in tumor vascular permeability are potential determinants of response and resistance to sunitinib in HCC.

Jarnagin *et al.*^[21] reports the results of 34 patients (26 intrahepatic cholangiocarcinoma and eight HCC) who received hepatic arterial infusion with floxuridine and dexamethasone. Patients with high pretreatment AUC had a lon-

Table 1 Summary of different hepatocellular carcinoma treatment, dynamic contrast-enhanced magnetic resonance imaging parameters and outcome

Ref.	Case number	Treatment	Parameter	Time interval	Outcome measure	P value
Wang <i>et al</i> ^[55] , 2004	7	Thalidomide	↓ Peak, ↓ Slope	8 wk	P <i>vs</i> NP	< 0.05
Liang <i>et al</i> ^[23] , 2007	19	Radiotherapy	↓ Peak, ↓ Slope	2 wk	R <i>vs</i> NR	< 0.05
Zhu <i>et al</i> ^[56] , 2009	34	Sunitinib	↓ K ^{trans}	2 wk	P <i>vs</i> NP	< 0.05
Jarnagin <i>et al</i> ^[21] , 2009	34 (26 ICC and 8 HCC)	Floxuridine (FUDR) and dexamethasone	High baseline AUC, ↓ K _{ep}	2 mo	OS	0.002
Yopp <i>et al</i> ^[57] , 2011	17 (14 and 3 HCC)	Floxuridine (FUDR) Bevacizumab	↓ AUC	2 wk	TTP	0.013 0.002
Hsu <i>et al</i> ^[58] , 2011	31	Sorafenib, TG/uracil	High baseline K ^{trans}	-	P <i>vs</i> NP	0.008
Hsu <i>et al</i> ^[58] , 2011	31	Sorafenib, TG/uracil	↓ K ^{trans}	2 wk	P <i>vs</i> NP	0.003
					OS	0.015
					PFS	0.030
Hsu <i>et al</i> ^[59] , 2012	67	Vandetanib	↓ K ^{trans}	1 wk	Pre <i>vs</i> Post	NS

HCC: Hepatocellular carcinoma; ICC: Intrahepatic cholangiocarcinoma; TG: Tegafur; P: Progression; NP: Non-progression; R: Responder. NR: Non-responder; PFS: Progression-free survival; OS: Overall survival; TTP: Time to progression; Pre: Pre-treatment; Post: Post-treatment; AUC: Area under curve; NS: Non-significant.

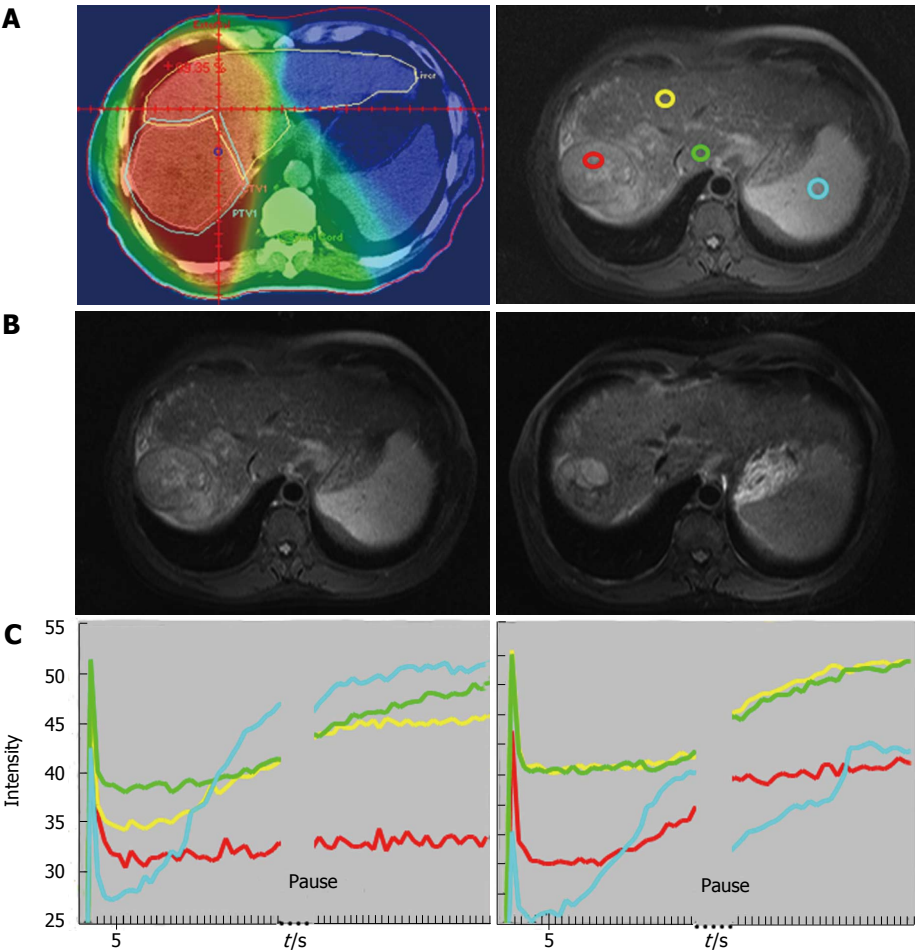


Figure 1 Forty-nine-year-old man with good local response. A: Isodose distribution and region of interests (ROIs): Left panel: Isodose distribution on center section of radiation treatment planning. Red: 45 Gy; orange: 40 Gy; yellow: 30 Gy; green: 15 Gy and blue: < 15 Gy. Right panel: ROIs on MRI before RT: red: tumor with strongest enhancement; yellow: non-tumor liver parenchyma receiving 30 Gy; green: non-tumor liver parenchyma receiving 15 Gy; blue: spleen; B: T1 weighted contrast-enhanced MRI before RT (left panel, the site corresponding to the right panel of (A) and after RT (right panel). Arrows indicate tumor margins; C: Time Intensity Curve of ROIs before RT (left panel) and at week 2 of RT (right panel). Red: tumor; yellow: 30 Gy; green: 15 Gy; blue: spleen. The curve of spleen is deviated after pause for respiration due to interference by lung perfusion. The initial spike due to refocusing artifact will not be counted into analysis. (From reference [23], reprint with permission).

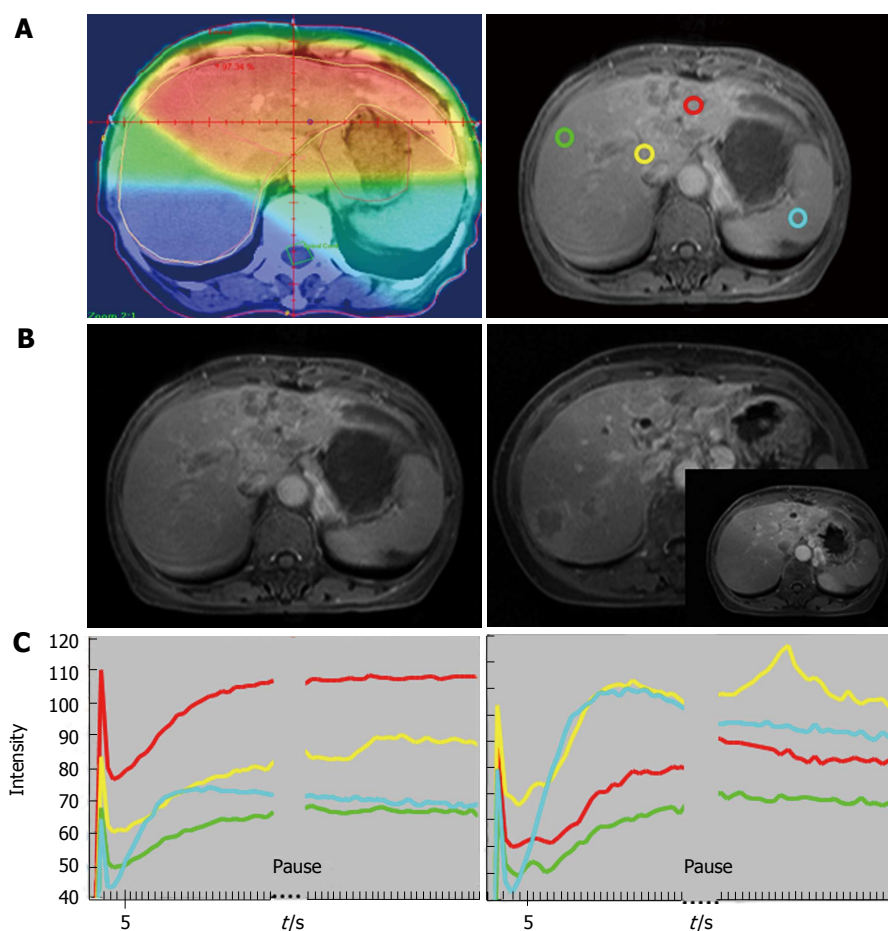


Figure 2 Sixty-four-year-old woman with good local response and intrahepatic recurrence outside of RT fields. A: Isodose distribution and region of interests (ROIs): Left panel: isodose distribution on center section of radiation treatment planning. Red: 45 Gy; orange: 40 Gy; yellow: 30 Gy; green: 15 Gy; blue: < 15 Gy. Right panel: ROIs on magnetic resonance imaging (MRI) before RT: red: tumor with strongest enhancement; yellow: non-tumor liver parenchyma receiving 30 Gy; green: non-tumor liver parenchyma receiving 15 Gy; blue: spleen; B: T1 weighted contrast-enhanced MRI before RT (left panel, same site as the right panel of (A) and after RT (right panel). The intersect picture of MRI over right panel demonstrates no tumor progression in the RT field on the center section of treatment planning. Arrows indicate tumor margins and arrowhead, recurrent tumor outside the field of RT; C: Time Intensity Curve of ROIs before RT (left panel) and at week 2 of RT (right panel). Red: tumor; yellow: 30 Gy; green: 15 Gy; blue: spleen. The initial spike due to refocusing artifact will not be counted into analysis. (From reference [23], reprint with permission).

ger median survival than those with low AUC ($P = 0.002$). Besides, decreased K^{trans} and K_{ep} on the first post-treatment MR scanning both predicted survival. Hence, pretreatment and early post-treatment changes in tumor perfusion characteristics may predict treatment outcome ahead.

Yopp *et al*^[57] evaluated 17 patients (14 intrahepatic cholangiocarcinoma and 3 HCC) treated with floxuridine and bevacizumab. Significant decreases in AUC and K^{trans} were noted in tumors after bevacizumab. Time to progression correlated inversely with changes in AUC after bevacizumab. Reductions in tumor perfusion were greater in tumors expressing markers of anti-hypoxia and VEGF.

In one study of locally advanced HCCs receiving sorafenib and cytotoxic therapy, conducted by Hsu *et al*^[58], a decrease of K^{trans} by 40% or greater after 14 days of treatment was correlated with longer progression free survival (PFS) and overall survival (OS). Besides, percentage of K^{trans} change (difference between pre- and post-treatment) is an independent predictor of tumor response, PFS, and OS (Figures 3 and 4). In another study, Hsu *et al*^[59] reported a randomized clinical trial of 67 HCC patients

with vandetanib treatment, but no significant vascular change was found 1 wk after treatment. They explained that the steady-state concentration of vandetanib will be reached after at least 4 wk of treatment. Besides, the vascular features of heterogeneous nature of HCC due to tumor necrosis, arterio-venous shunting within the tumors, and the effects of prior local therapy, might preclude a reliable MRI measurement and comparison.

HCC EVALUATED BY PERFUSION CT

Similar to DCE-MRI, perfusion CT imaging of the liver is performed by acquisition of serial images after contrast bolus injection to obtain various perfusion indices, including regional tumor blood flow, blood volume, flow-extraction product, and permeability-surface area product. Previous reports have suggested that CT perfusion parameters can be used for quantifying tumor vascularity^[60-64] and angiogenesis^[65] in HCC, or as biomarkers to monitor response to chemoembolization^[50], chemotherapy and a range of different targeted agents^[66-68]. For ex-

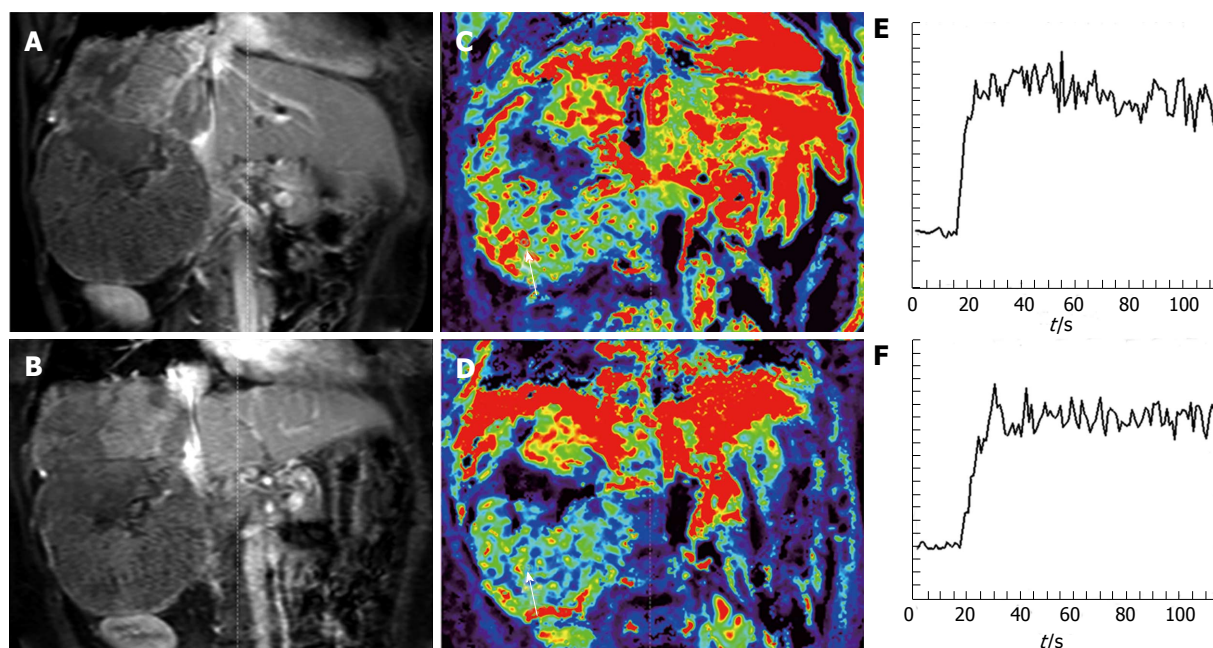


Figure 3 Representative dynamic contrast-enhanced magnetic resonance imaging findings in one advanced hepatocellular carcinoma patient. A: Post-contrast T1-weighted magnetic resonance imaging at baseline; B: After 14 d of study treatment; C: Corresponding color K^{trans} maps at baseline; D: After 14 d of study treatment. Hypervascular area was indicated by red color. The selected region of interest for K^{trans} measurement was indicated by white arrows. In this patient, the K^{trans} values at baseline and after study treatment were $798.6 \times 10^{-3}/\text{min}$ and $206.6 \times 10^{-3}/\text{min}$, respectively; E: The initial area under the gadolinium concentration-time curves (IAUC) at baseline; F: After study treatment from the same patient. The IAUC values at baseline and after study treatment were $1526.2 \text{ mmol/kg} \times \text{s}$ and $1376.1 \text{ mmol/kg} \times \text{s}$, respectively. (From reference [58], reprint with permission).

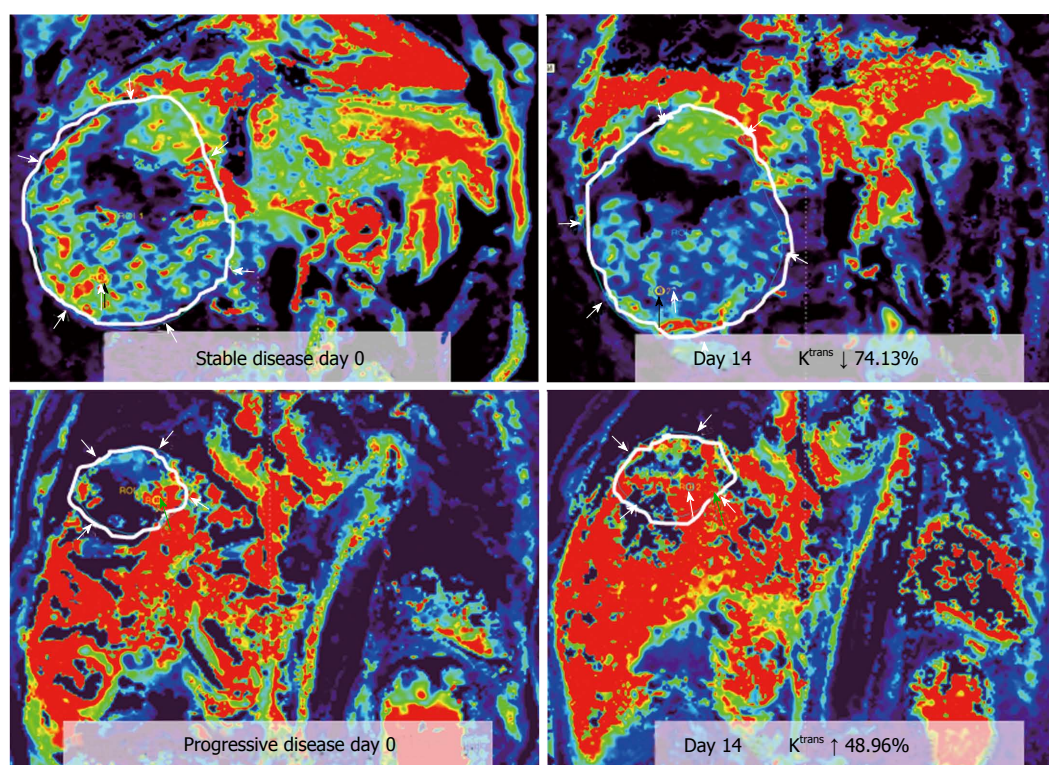


Figure 4 Representative dynamic contrast-enhanced magnetic resonance imaging K^{trans} color maps before treatment (day 0, left hand side) and day 14th after treatment (right hand side) in two advanced hepatocellular carcinoma patients. Corresponding hypervascular area was indicated by red color. region of interests analysis is more sensitive based on hypervascular part than entire tumor, with mean values. K^{trans} is a good diagnostic biomarker in differentiation between stable disease (SD, upper row) and progressive disease (PD, lower row) in two patients with hepatocellular carcinoma. Difference of K^{trans} (ΔK^{trans}) between SD and PD measured on hypervascular part and entire tumor are both significant. (From reference [58], reprint with permission).

ample, in one study of locally advanced HCCs receiving bevacizumab and cytotoxic therapy, high pretreatment K^{trans} by perfusion CT indicated those patients with a RECIST response^[67]. Their findings were comparable with the results investigated by Hsu *et al.*^[58]: in patients with locally advanced HCCs receiving sorafenib and cytotoxic therapy, high pre-treatment K^{trans} measured by DCE-MRI indicated those patients who did not develop progressive disease^[58]. The main drawback of perfusion CT is radiation exposure, but recent advances in multidetector CT technology many help achieve acceptable radiation dose in HCC patients.

FUTURE DIRECTION

Dynamic contrast-enhanced MR imaging is a reproducible technique. According to previous studies, the reproducibility of K^{trans} is good to moderate (coefficient of repeatability ranges from about 15%-40%)^[69,70]. This suggests that in a well-conducted study, a change of K^{trans} value of more than 40% is likely to indicate a significant drug effect^[71]. The reproducibility of DCE-MR imaging parameters is influenced by lesion location, with the parameters being significantly more reproducible in the liver than in the lung^[72]. However, current DCE-MRI technique lacks standardization across multiple MR platforms and institutions, making it difficult to implement the technique in a multicenter setting^[17,73]. Besides, there is a need to establish clear thresholds for a significant response when using quantitative DCE-MR imaging parameters for assessment of therapy response.

CONCLUSION

DCE-MRI is an imaging technique that appears to provide quantitative and biologically relevant informations related to tumor vasculature and angiogenesis, which can inform novel drug efficacy, monitor treatment response and act as an imaging biomarker to predict treatment outcome and survival in HCC patients.

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WJG 20th Anniversary Special Issues (1): Hepatocellular carcinoma

Development of systemic therapy for hepatocellular carcinoma at 2013: Updates and insights

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combinational approaches. Future directions for testing innovative systemic agents for HCC will also be discussed.

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Key words: Liver neoplasms; Systemic treatment; Biologics; Staging; Clinical trial

Core tip: This review article aims to provide an update and vision on the development of novel targeted agents for liver cancers. Recently released phase III clinical trial results as well as important future focus will be discussed.

Abstract

A growing number of multi-targeted tyrosine kinase inhibitor (TKI) has undergone testing for hepatocellular carcinoma (HCC). Unfortunately, this enthusiasm has recently been discouraged by a number of negative phase III studies on several anti-angiogenic TKIs in HCC. Several postulations have been made to account for this phenomenon, namely the plateau effects of anti-angiogenesis approach, the heterogeneity of HCC in terms of background hepatitis/cirrhosis and tumor biology, as well as the way how clinical trials are designed. Regardless of the underlying reasons, these results suggested that alternative strategies are necessary to further develop systemic therapy for HCC. Several new strategies are currently evaluated: for examples, molecular agents with activities against targets other than vascular endothelial growth factor receptor are being evaluated in on-going clinical trials. In addition, different approaches of targeted agents in combination with various treatment modalities, such as concurrently with another molecular agent, cytotoxic chemotherapy or transarterial chemoembolization, are being developed. This review aims to give a summary on the results of recently released clinical trials on TKIs, followed by discussion on some of the potential novel agents and

Chan SL, Yeo W. Development of systemic therapy for hepatocellular carcinoma at 2013: Updates and insights. *World J Gastroenterol* 2014; 20(12): 3135-3145 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i12/3135.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i12.3135>

INTRODUCTION

Following the approval of sorafenib for treatment of unresectable hepatocellular carcinoma (HCC), there has been a surge of interests in the clinical development of targeted agents for HCC. Despite intensive efforts being put on drug testing over the past 5 years, the outcomes of patients with advanced HCC remain poor. Recently, a number of novel multi-targeted tyrosine kinase inhibitors (TKIs) have completed phase III testing but all of the results have turned out to be negative^[1-3]. In addition, these large scale clinical trials persistently reported a median overall survival (OS) of 9 to 10 mo, indicating that the benefit of existing panel of TKIs has reached a plateau. Although these negative results appear discouraging to some, experience from these trials have shed insights to

Table 1 Results of completed phase III clinical trial on systemic agents for hepatocellular carcinoma

Clinical trial	Drug	Population	TTP (mo)	OS (mo)
1st line				
SHARP ^[89]	Sorafenib	19% HBV; 29% HCV	5.5	10.7
	Placebo	18% HBV; 27% HCV	2.8	7.9
Asian SHARP ^[90]	Sorafenib	71% HBV; 11% HCV	2.8	6.5
	Placebo	78% HBV; 4.0% HCV	1.4	4.2
SUN1170 ^[11]	Sunitinib	55% HBV; 21% HCV	4.1	8.1
	Sorafenib	53% HBV; 22% HCV	3.8	10.0
BRISK-FL ^[2]	Brivanib	44% HBV; 20% HCV	4.2	9.5
	Sorafenib	45% HBV; 21% HCV	4.1	9.9
Linifanib <i>vs</i> sorafenib ^[12]	Linifanib	49% HBV	5.4	9.1
	Sorafenib		4.0	9.8 (<i>P</i> = NS)
SEARCH ^[57]	Sorafenib + Erlotinib	NA	3.2	9.5
	Sorafenib		4.0	8.5 (<i>P</i> = 0.2)
Chemotherapy ^[62]	Doxorubicin	80% HBV; 8% HCV	NA	6.8
	PIAF	82% HBV; 4% HCV		8.7
2nd line				
BRISK-PS ^[3]	Brivanib	NA	4.2	9.4
	Placebo		2.7	8.2
			(<i>P</i> = 0.0001)	(<i>P</i> = 0.33)

HBV: Hepatitis B virus; HCV: Hepatitis C virus; NA: Not available; OS: Overall survival; TTP: Time to progression; NS: Non-significant; HCC: Hepatocellular carcinoma.

the design of new approaches in drug testing. In the current paper, we aim to give an update on the recent data on clinical trials using molecular targeted agents and discuss some of novel approaches for developing systemic agents for HCC.

UPDATE OF RESULTS OF CLINICAL TRIALS AT 2013

Phase III studies on anti-angiogenic TKIs

Sorafenib is a small molecule TKI which targets at multiple receptor kinases and signaling molecules, namely the vascular endothelial growth factor receptor (VEGFR), platelet derived growth factor receptor (PDGFR), B-Raf, Fms-related tyrosine kinase (FLT) and c-kit at nanomolar concentration^[4,5]. Sorafenib is generally considered an anti-angiogenic agent though its exact mechanism remains unclear. Following the positive results of clinical trials using sorafenib, a number of multi-targeted angiogenic TKIs have undergone clinical testing for the treatment of advanced HCC. These TKIs are characterized by their abilities to inhibit a wide spectrum of membranous receptors, mainly including VEGFR, FGFR (fibroblast

growth factor receptor) and PDGFR. The key results of completed phase III clinical trials on these agents are summarized in Table 1.

Sunitinib is the first anti-angiogenic TKI to compare with sorafenib in phase III trial. The targets of sunitinib include VEGFR, PDGFR, c-kit and FLT-3. Despite multiple targets of sunitinib, clinical experiences with the drug suggest that anti-angiogenesis is probably the major anti-neoplastic mechanism. Initial phase II studies reported potential activities of sunitinib in advanced HCC, with disease control rate ranging from 38% to 52%, and overall survival ranging from 8.0 to 9.8 mo^[6,7]. To validate the results of these studies, a multi-centered phase III clinical trial (SUN1170) was launched in 2008 to compare sunitinib to sorafenib as the first-line treatment for advanced HCC. The original design was aimed to recruit 1200 patients with randomization in 1:1 ratio into two arms, namely sunitinib 37.5 mg daily or sorafenib 400 mg twice daily. However, after accruing 1074 patients, the study was prematurely stopped when a preplanned safety analysis revealed a higher incidence of serious adverse events in the sunitinib arm^[1]. The primary endpoint, OS, of the sunitinib arm was 7.9 mo, which was significantly worse than the 10.2 mo in the sorafenib arm (*P* = 0.0014), while the time-to-progression (TTP) was similar between the two arms (sunitinib 4.1 *vs* sorafenib: 3.8 mo; *P* = 0.8312)^[1]. In terms of toxicity profile, sunitinib was associated with more grade 3 or above complications, including bleeding events (11.4%), thrombocytopenia (29.7%) and neutropenia (25.7%). The toxicity and inferior outcomes of patients treated with sunitinib have stopped further development of the agent in HCC.

Brivanib is a dual VEGFR and FGFR inhibitor^[8]. In preclinical models, the drug has been shown to have more potent anti-angiogenic effects than sorafenib, and the additional activity against FGFR is postulated to counteract the resistance mechanism to angiogenic agents targeting VEGF alone. In phase II clinical trials, brivanib has demonstrated reasonable activity in both first- and second-line setting with TTP of 2.8 and 1.4 mo, respectively^[9,10]. Two randomized phase III clinical trials were conducted to assess the agent in the first-line (BRISK-FL) and second-line (BRISK-PS) settings. BRISK-FL is a head-to-head randomized phase III clinical trial comparing brivanib to sorafenib as the first-line therapy in patients with unresectable HCC. The study enrolled 1155 patients who had not received any prior systemic treatment, and participants were randomized in 1:1 ratio to receive brivanib at 800 mg daily or sorafenib at 400 mg twice daily, with OS as the primary endpoint^[2]. The clinical trial has adopted a non-inferiority study design. According to the latest publication, the primary endpoint, OS non-inferiority between brivanib and sorafenib, was not met [brivanib: 9.5 mo; sorafenib: 9.9 mo; HR = 1.06; *P* = non-significant (NS)]^[2]. There were also no difference in TTP between brivanib and sorafenib (brivanib: 4.2 mo; sorafenib: 4.1 mo; *P* = NS)^[2]. In addition, brivanib appeared to be less well tolerated than sorafenib, as evidenced by higher rates of adverse events resulting in

Table 2 List of selected ongoing clinical trials on novel targeted therapy for hepatocellular carcinoma

Drug	Design	Phase	Status	NCT number
Single-agent TKI				
Dovitinib (TKI258)	Dovitinib <i>vs</i> sorafenib (1 st line)	Randomized phase II	Completed	NCT01232296
Carbozantinib	Carbozantinib <i>vs</i> placebo (2 nd line)	Phase III	Ongoing	NCT01908426
c-MET inhibitor				
Tivantinib	Tivantinib <i>vs</i> placebo (2 nd line)	Phase III	Ongoing	NCT01755767
INC280	INC280 (1 st line in c-MET expressing HCC)	Phase I / II	Ongoing	NCT01737827
Oncolytic poxvirus				
JX594	JX594 <i>vs</i> placebo (2 nd line)	Randomized phase II	Ongoing	NCT01387555
Glypican-3				
GC33	GC33 <i>vs</i> placebo (2 nd line)	Phase III	Completed	NCT01507168
mTOR inhibitor				
Everolimus	Everolimus <i>vs</i> placebo (2 nd line)	Phase III	Press release	NCT01035229
Temsirolimus	Temsirolimus (1 st or 2 nd line)	Phase II	Abstract	NCT01251458
CC-223	CC-223 in solid tumors including HCC	Phase I / II	Ongoing	NCT01177397
Arginine deprivation therapy				
ADI-PEG 20	ADI-PEG 20 <i>vs</i> placebo (2 nd line)	Phase III	Ongoing	NCT01287585
Combination				
Sorafenib	Sorafenib + doxorubicin <i>vs</i> sorafenib	Phase III	Accrual	NCT01015833
Sorafenib	TACE + sorafenib <i>vs</i> TACE (ECOG 1208)	Phase III	Accrual	NCT01004978
Everolimus	TACE + everolimus <i>vs</i> TACE	Randomized phase II	Accrual	NCT01379521
Axitinib	TACE + axitinib	Phase II	Accrual	NCT01352728
Bevacizumab and Erlotinib	Bevacizumab + erlotinib <i>vs</i> sorafenib	Randomized phase II	Accrual	NCT01180959

TACE: Transarterial chemoembolization; TKI: Tyrosine kinase inhibitor; HCC: Hepatocellular carcinoma; c-MET: c-mesenchymal-epithelial transition factor-1.

treatment discontinuation (brivanib: 43% *vs* sorafenib: 33%)^[2]. In the second-line setting, BRISK-PS compared brivanib to placebo in patients who were refractory or intolerant to first-line treatment of sorafenib. The trial has randomized 395 patients in 2:1 ratio to receive brivanib 800 mg daily or placebo along with best supportive care, with OS as the primary endpoint. Disappointingly, although TTP was significantly longer in the brivanib arm than placebo (4.2 *vs* 2.7 mo; $P = 0.0001$), providing a signal of potential activity of brivanib, the study failed to reach its primary endpoint of achieving benefit in OS

(brivanib: 9.4 *vs* placebo: 8.2 mo; $P = 0.33$)^[3].

Linifanib is an oral TKI with selective activity against VEGFR and PDGFR. Preclinical studies have reported potent activity of the agent on HCC xenografts. In a single-arm phase II study, linifanib was associated with a radiologic response rate of 9.1% and median TTP of 3.1 mo^[11]. These promising results have led to an international multi-centered phase III trial comparing linifanib to sorafenib. In this trial, a total of 1035 patients were randomized to linifanib at 17.5 mg daily or sorafenib at 400 mg twice daily. According to the preliminary results released at American Society Clinical Oncology (ASCO) Gastrointestinal Cancers Symposium in 2013, linifanib failed to demonstrate superiority or non-inferiority in terms of OS when compared with sorafenib (linifanib: 9.1 mo; sorafenib: 9.8 mo; $P = \text{NS}$)^[12].

Following the results of these studies, both brivanib and linifanib were generally considered not to be valid options for patients with advanced HCC.

EMERGING MOLECULAR TARGETS

In addition to the anti-angiogenic multi-targeted TKIs, there is a growing number of biologics that target at different molecular pathways. Some of these treatments act along molecules of intracellular signaling pathways while others are agents relying on the inhibition of non-signaling dependent mechanism (highlighted in Table 2). A number of agents have shown promising preliminary data for HCC, and these have been selected for more detailed discussion below.

c-mesenchymal-epithelial transition factor-1 inhibitor

c-mesenchymal-epithelial transition factor-1 (c-MET) is a membrane receptor that is essential for hepatocyte and tissue remodeling of liver after hepatic injury^[13,14]. The activation of c-MET is implicated in the proliferation, invasion and metastases of cancer cell^[15,16]. The expression of c-MET receptor protein occurs in 20% to 48% of human HCC samples^[17-20], and has been shown to be a poor prognostic factor in patients with HCC. In addition, the inactivation of c-MET could lead to regression of tumors in xenograft model and growth inhibition in HCC cell lines^[21]. Therefore, therapeutics aiming at the c-MET receptor is a rational approach for HCC. Two agents, namely tivantinib and cabozantinib, have undergone more advanced development. Tivantinib is an oral tyrosine kinase inhibitor of c-MET^[22]. A randomized phase II trial comparing the use of tivantinib *vs* placebo as the second-line treatment, showed that the TTP was slightly improved in the tivantinib arm (Tivantinib 1.6 mo; placebo 1.4 mo; $P = 0.04$). In particular, a more obvious improvement of TTP was noted in patients with tumors overexpressing c-MET (Tivantinib arm: 2.7 mo; placebo arm: 1.4 mo; HR = 0.38, 95%CI: 0.18-0.81, $P = 0.01$)^[23]. Currently, a phase 3 study is underway to compare tivantinib *vs* placebo in subjects with c-MET overexpressing HCC who have failed one prior systemic therapy (NCT01755767).

On the other hand, carbozantinib is an oral TKI with

activity against both c-MET and VEGFR-2. In a phase II randomized discontinuation clinical trial; patients were treated with cabozantinib and reassessed at 12 wk. Those patients with evidence of response would continue with cabozantinib while patients with stable disease were randomly assigned to cabozantinib or placebo. According to the results reported in the 2012 ASCO meeting, an impressive efficacy has been observed; the progression-free survival (PFS) was 4.4 mo while the median OS was 15.1 mo in the cabozantinib arm^[24]. This encouraging data has led to a planning of a phase III clinical trial testing the efficacy of cabozantinib in the second-line setting (NCT01908426). This phase III study is also planning to collect the tumor tissues to determine whether c-MET is a predictive marker, an aspect that has not been studied in the previous phase II study.

mTOR inhibitor

The phosphoinositide 3-kinase (PI3K)/Akt/mTOR axis is involved in multiple cellular processes including survival and proliferation^[25]. This signaling is initiated when membrane receptors are activated by binding of growth factors, which in turn recruit and activate the PI3K. The activation of PI3K will lead to a cascade of activation of downstream effectors leading to activation of mTOR. Comprehensive genomic analyses have shown that components of the PI3K/Akt/mTOR pathway are frequently deregulated in up to 50% of HCC^[26,27]. Therefore, targeting the components of this pathway, especially the downstream molecule mTOR, has been a research focus for development of therapeutics for HCC.

mTOR inhibitors, especially everolimus and temsirolimus, are being investigated in patients with HCC. In a phase I study of everolimus in 28 patients with advanced HCC, of whom over 70% were treated with more than one prior regimen, the maximum tolerated dose was 10 mg daily. At this dose, treatment with everolimus yielded a disease control rate of 44% and an overall survival of 8.4 mo^[28]. The drug was generally well tolerated with most common toxicities being fatigue and hyperglycemia^[28]. A phase III study comparing everolimus with placebo (EVOLVE-1) in patients who have failed or become intolerant to sorafenib has recently been completed (NCT01035229). At the time of writing, there has been a press release indicated that the EVOLVE-1 study failed to reach its primary endpoint of extending OS with everolimus^[29]. Further detailed results are expected in the near future. For temsirolimus, a phase I / II study in a heavily pretreated population of unresectable HCC has reported the maximum tolerated dose (MTD) of temsirolimus to be 25 mg every week; amongst the 36 patients recruited in the phase II portion, the disease control rate was 38.9%^[30,31]. A number of clinical trials have been designed to evaluate the combination of mTOR inhibitor with sorafenib (see section below: combinational treatment approach). Another mTOR inhibitor, CC-223, which possesses dual activity against mTORC1 and 2, is also undergoing phase I / II development in solid tumors including HCC (NCT 01177397).

Histone deacetylase inhibitor

The expression of tumor suppressor genes is influenced by coiling and uncoiling of DNA around histone, which is mainly mediated by histone acetylation. Acetylation of histone results in less condensed chromatin leading to expression of gene expression while histone deacetylases (HDACs) remove the acetyl groups from histones leading to condensed and transcriptionally silenced chromatin^[32]. Such histone modification is one of the major epigenetic mechanisms on gene regulation, and the HDACs are amenable to inhibition by HDAC inhibitors. This class of agents was initially investigated for hematological malignancies, and vorinostat and romidepsin have been approved for the treatment of peripheral T-cell lymphoma^[33,34].

For HCC, preclinical studies showed that treatment with HDAC inhibitor could induce apoptosis in HCC cell lines^[35-37]. A phase I / II clinical trial assessed HDAC inhibitor, belinostat, for treatment of advanced HCC. Amongst the 42 patients treated in the phase II portion, reasonable efficacy was demonstrated in a heavily pretreated population, with disease stabilization rate of 47.6% and PFS of 2.64 mo^[38]. Belinostat was well tolerated with lower than 10% grade 3 or above toxicities^[38]. More interesting findings come from the exploratory analysis on the role of HR23B to predict the clinical response. HR23B is a protein which is responsible for shuttling ubiquitinated cargos for proteosomal degradation. It has been suggested that the expression of HR23B is a potential predictive marker for response to HDAC inhibitor in hematological malignancies^[39,40]. In the aforementioned phase II trial on HCC, it was shown that tumors with high HR23B histoscores is associated with a higher rate of disease stabilization ($P = 0.036$)^[38]. Further studies are required to study the clinical role of HR23B as predictive biomarker in HCC.

Arginine deprivation therapy

In human cells, arginine is a non-essential amino acid, and arginine is synthesized from citrulline through a series of enzymatic reactions^[41]. However, HCC cells are known to be defective of a number of these enzymes including argininosuccinate synthetase (ASS) or ornithine transcarboxylase (OTC); as a result, there is impairment in the cellular ability to replenish the arginine once it is depleted, which subsequently leads to cell death^[42,43]. This mechanism of arginine deprivation is attractive because it could provide selective cytotoxic effect on tumor but not non-tumorous tissues.

At present, two classes of arginine degrading enzymes have undergone clinical testing, namely the ADI-PEG 20 and the PEG-BCT-100. ADI-PEG 20 is an arginine deaminase which depletes arginine level by converting it to citrulline and ammonia. Two phase II studies have been completed in HCC^[44,45]. The reported disease-control rate (DCR) and the mean OS were in the range of 30%-60% and 7-16 mo, respectively. This has led to the conduct of an international multi-centered study to compare the efficacy of ADI-PEG 20 vs placebo in the second-line set-

ting, following failure to sorafenib (NCT01287585).

PEG BCT-100 is a recombinant human arginase which degrades arginine by converting it to ornithine and urea^[46-49]. Compared to ADI-PEG 20, the agent has a theoretical advantage of having broader activity on HCC cells which express ASS but not OTC. Clinical trials in phase II setting are being planned.

Immunotherapy

HCC is an inflammation-associated cancer; analysis of the tumor microenvironment has suggested that local immune responses may be prognosticator of the disease^[50]. Specific anti-tumor T-cell responses can be detected in patients with HCC. Immune responses are regulated by molecules that provide co-stimulatory and inhibitory signals to T cells. Down regulation of T-cell activity upon binding to ligands on antigen-presenting cells and tumor cells affects peripheral tolerance and protection from autoimmune damage^[51]. The recent approval of ipilimumab for patients with melanoma and Sipuleucel-T for patients with prostate cancer, has highlighted the possibility of adopting immunotherapy in other malignancies including HCC^[52,53].

COMBINATIONAL TREATMENT APPROACH

The concept of combination of different agents or treatment modalities is attractive for the following reasons^[54]: (1) taking into consideration most of the single-agent therapies are associated with low radiologic response rate and the high HCC tumor heterogeneity, the concurrent use of compounds with synergistic activity may potentially improve the clinical outcome; and (2) the survival time of patients with advanced HCC is relatively short compared with other solid tumors, thus limiting the possibility of sequential treatments using individual agents. Obviously, one of the biggest obstacles for combination treatment is the concomitant compromised hepatic reserves present in most HCC patients with most of them suffering from cirrhosis. Therefore, carefully planned and dedicated early clinical trials are warranted to investigate the toxicity and efficacy of novel combinations in patients before proceeding to phase III development. Over the past few years, different ways of combinational treatment has been explored by various groups, and these are discussed below.

Combination of targeted agents

Most of developments have been based on combination of a novel class of targeted agent with sorafenib. At present, there are more than 20 clinical trials with such design. According to the recently available results, it appears that the difficulty of combining sorafenib with other targeted agent may be greater than expected. For examples, in a phase I / II study testing the combination of temsirolimus and sorafenib, the MTD of the combinational regimen was sorafenib at 200 mg twice daily and temsirolimus at 10 mg weekly, which was lower than that found in melanoma patients with hepatic dysfunction^[55].

In another phase I study on sorafenib and everolimus, the MTD of everolimus was only 2.5 mg daily, which was a significantly lower dosage than that required to achieve a biologically effective dose in human body^[56]. Similar problem is also experienced in the phase III SEARCH study comparing sorafenib-erlotinib *vs* sorafenib-placebo^[57]. In this trial, not only did the sorafenib-erlotinib not improve clinical outcomes, the combination was associated with shorter duration of treatment and higher withdrawal rate indicating poor tolerance.

There have been fewer studies on the combination with a non-sorafenib agent. At present, the most well studied regimen is the combination of erlotinib and bevacizumab. In a phase II single-arm study of 40 Caucasian HCC patients, Thomas *et al*^[58] reported a response rate of 25%, and a median PFS and OS of 9.0 mo and 15.7 mo respectively in an initial report. The results were subsequently updated in a final analysis, which demonstrated a median PFS and OS of 7.2 mo and 13.7 mo^[59]. However, another phase II study with the same combination failed to reproduce the survival data; the response rate was only 3.7% and the overall survival was 9.5 mo^[60]. A randomized phase II study comparing bevacizumab-erlotinib to sorafenib is currently underway to validate the efficacy of this combinational regimen (NCT01180959).

Chemotherapy plus targeted agent

Although chemotherapy has not been directly compared to placebo or sorafenib in randomized studies, chemotherapy has persistently been associated with a high radiologic response and a large magnitude in decrease of serum alpha-fetoprotein level^[61-63]. The recently published phase III data on EACH study comparing FOLFOX4 to doxorubicin chemotherapy has also suggested that FOLFOX chemotherapy is a potential option of systemic treatment for patients with advanced HCC, with radiologic response of over 8%^[64]. Theoretically, the addition of chemotherapy could overcome the weakness of cytostatic property of molecular targeted agents. To test this hypothesis, a randomized phase II clinical trial has been conducted to compare sorafenib (400 mg twice daily)-doxorubicin (60 mg/m² every 3 wk) combination *vs* doxorubicin (60 mg/m² every 3 wk). According to the trial results, there was an improvement of both OS (13.7 *vs* 6.5 mo; *P* = 0.006) and radiologic response rate (62% *vs* 29%) favoring the combination arm^[62]. However, this benefit was at a cost of increased toxicities in the combinational arm especially with increased rate of left ventricular systolic dysfunction (all grade 19% *vs* 2%). It remains unclear whether the cardiac toxicity is due to drug interaction or due to the synergistic toxicity conferred by VEGF inhibition with sorafenib. A phase III clinical trial is currently undertaken to study the efficacy and safety of the sorafenib-doxorubicin combination *vs* single-agent sorafenib in the first-line setting (NCT01015833).

Transarterial chemoembolization plus targeted agent

HCC is a highly vascular tumor and transarterial chemoembolization (TACE) could induce tumor hypoxia, there-

by provoke a post-treatment surge of angiogenic factors including VEGF that may occur as early as a few hours post TACE. The event may contribute to the revascularization of tumors and reduction of the efficacy of TACE^[65,66]. In addition, the peripheral rim of HCC tumors frequently escapes the cytotoxic effects of TACE because of tumor repopulation, and microscopic tumor progression is frequent during the interval between each treatment cycle of TACE^[67]. Combining anti-angiogenic drugs with TACE may potentially improve treatment outcomes^[68].

The concept of combining sorafenib and TACE was initially tested in a single arm phase II study in which sorafenib was started at 1 wk after TACE with drug-eluting beads. This reported a DCR of 95% and objective response rate of 58% according to European Association for the Study of the Liver criteria^[69]. However, the global SPACE study, designed to test the continuous administration of sorafenib during TACE, failed to demonstrate significant benefit favoring the combinational approach. In the clinical trial, patients were randomized into two arms: one arm undergoing continuous administration of sorafenib 400 mg twice daily together with TACE at specified intervals and another arm receiving placebo and TACE. The primary endpoint was time to radiologic progression (TTRP). According to the results released in the 2012 ASCO Gastrointestinal Cancers Symposium, the study has met its primary endpoint on the improvement of TTRP in the sorafenib arm as compared to placebo arm [median TTRP of sorafenib = 169 d *vs* placebo = 166 d; HR = 0.797, *P* = 0.072 (pre-specified *P* value for the one-sided Log-rank test was set at 0.15)]^[70]. However, there was no statistically significant difference in OS and response rate between the two arms. In view of the small difference in the TTRP and the lack of difference in OS, most of the clinicians do not consider the results of this trial to be encouraging.

The less impressive results of SPACE clinical trial have casted shadow on whether the combination of TACE and sorafenib is an effective approach. Other groups attempt to address the issue with different studies. For examples, a multi-centered phase III ECOG 1208 study is underway, testing the continuous use of sorafenib with TACE *vs* placebo (NCT01004978). This phase III clinical trial has very similar design to the SPACE trial. The clinical trial may help further determine whether the approach of concurrent administration of sorafenib together with TACE is effective for treatment of HCC. On the other hand, we are conducting a phase II clinical trial testing the use of axitinib in combination with TACE (NCT01352728). Axitinib is a more potent TKI of all three VEGFRs1-3, and its use could potentially inhibit the surge of VEGF levels after TACE at a greater extent than sorafenib. The clinical trial is expected to complete accrual in early 2014.

came from subgroup analyses: both geographical difference and hepatitis status have had significant effects on treatment outcomes^[71]. Patients with hepatitis C virus infection or patients of non-Asian ethnicity tend to derive more benefits from sorafenib than patients with hepatitis B virus or the Asian origins. This type of finding was also similarly observed in the subgroup analysis of the Asian SHARP trial^[71]. Different explanations including genetic background, molecular pathogenesis, aggressive approach using surgery/locoablative treatment between West and East, have been postulated. Regardless of the underlying postulations, the geographical location and the hepatitis status should be taken into consideration during the design of clinical trials in HCC. Preferably, a dedicated phase I / II clinical trial should be designed to evaluate new agents in hepatitis B and hepatitis C-related HCC subpopulations, in addition, the design of international multi-centered trial should consider stratification by geographical regions, in terms of East *vs* Non-East, in the randomization process.

Selection of suitable patients

It is evident that unresectable HCC population consists of a highly heterogeneous group of patients with a wide spectrum of survival ranging from a few months only to longer than 2 years^[72-74]. As a result, it is difficult to precisely estimate the survival of patients during the design of clinical trials that encompass a heterogeneous population. Different staging systems have been developed to define suitable patients for the administration and testing of systemic agents. At this juncture, the Barcelona Cancer Liver Clinic (BCLC) classification is the most frequently used staging system for clinical trials. It has to be noted that BCLC was initially designed for allocation of treatment rather than for prognostication of HCC. As a result, the staging system is suboptimal in identifying homogeneous group of patients in terms of prognosis and disease behavior. For examples, patients classified as BCLC stage C disease (*i.e.*, advanced disease defined as patients with Child's A or B liver function, having a performance status of 1 or above, and the presence of vascular invasion or extra-hepatic disease) has been assigned the target group for testing systemic agents. However, there have been studies suggesting that the BCLC system is inadequate in predicting the short-term outcome of patients or identifying a homogenous group of patients with advanced disease^[75,76]. Also, the treatment allocation as recommended by BCLC is considered too conservative by most Asian clinicians. For examples, most of the hepatobiliary cancer surgeons in Asia will not regard invasion of branch of portal vein as a definitive contraindication to surgical resection^[77,78]. In view of these limitations, a more precise staging system is necessary to identify a homogenous group of patients for testing systemic agents.

On the other hand, in Asia, because of the limited choice and the low efficacy of available systemic agents, patients with unresectable HCC confined to liver are often treated with multiple cycles of TACE before consid-

FUTURE DIRECTIONS

Design of clinical trial

One interesting point observed from the SUN1170 study

ering systemic agents, albeit limited efficacy^[79]. Given the increasing number of novel agents currently being tested that may potentially improved efficacy for HCC, studies are indicated to refine the TACE population and define the optimal timing to shift away from TACE when the treatment is no longer effective. For examples, there has been a recent study by the European group on the development of a scoring system to guide the retreatment with transarterial chemoembolization^[80].

Personalized treatment

Experiences from the lung and breast cancer fields have shown that success in clinical trials using targeted agents can only be improved if we are able to apply to appropriately selected patients whose tumors are “addicted” to a known driver gene or pathway. An ideal approach would be targeting individual agents in patients whose HCC tumors have the corresponding genetic mutations. With recent genomic sequencing showing that a genetic driver mutation, if present, occurs at a rate of lower than 5% in HCC, the chance of picking up a responder of a novel agent in an unselected population is much lower than 5%^[81-85]. This clinical challenge is evidenced by the persistently low response rate observed in multiple clinical trials on molecular targeted agents in unselected HCC populations, all of which have resulted in an overall survival that leveled off in the range of 9 to 10 mo (Table 1). Given the reported data on the role of c-MET expression and the potential use of HR23B to predict response of individual targeted agents, future clinical trials should be tailored towards identification of molecularly enriched patient population. Therefore, it is important to obtain pre-treatment tumor samples in the conduct of clinical trials. Owing to the invasive nature of tumor biopsy, a number of groups are currently studying the use of massive parallel sequencing to study the cancer genome in patients’ plasma samples, which could potentially obviate the need of needle biopsy^[86-88].

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WJG 20th Anniversary Special Issues (3): Inflammatory bowel disease

Immunosuppressive therapies for inflammatory bowel disease

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Abstract

Inflammatory bowel disease (IBD) is comprised of Crohn's disease and ulcerative colitis, both chronic inflammatory intestinal disorders of unknown etiology characterized by a waxing and waning clinical course. For many years, the drug therapy was limited to sulfasalazine and related aminosalicylates, corticosteroids and antibiotics. Studies suggesting that the pathophysiology of these disorders relates to a dysregulated, overactive immune response to indigenous bacteria have led to the increasing importance of immunosuppressive drugs for the therapy of IBD. This review details the mechanisms of action, clinical efficacy, and adverse effects of these agents.

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Key words: Crohn's disease; Ulcerative colitis; Inflammatory bowel disease; Immunosuppressives; Tumor necrosis factor inhibitors

Core tip: This manuscript reviews the current status of immunosuppressive therapy for inflammatory bowel disease. It describes the mechanism of action, clinical efficacy and adverse effects of immunomodulators including azathioprine, 6-mercaptopurine, methotrexate, and cyclosporine and biologics including anti-tumor necrosis factor (TNF) agents and adhesion molecule inhibitors. It emphasizes the role of azathioprine, 6-mercaptopurine, and methotrexate in the long-term maintenance of Crohn's disease, the utility of cyclosporine in severe refractory ulcerative colitis and the unique role of anti-TNF agents in the remission induction and maintenance of difficult to treat patients with Crohn's disease and ulcerative colitis.

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INTRODUCTION

Inflammatory bowel disease (IBD) includes Crohn's disease and ulcerative colitis, both chronic inflammatory disorders of the gastrointestinal tract, characterized by a relapsing and remitting course^[1]. In the United States, the incidence of Crohn's disease is estimated to be between 6-8 per 100000, with a prevalence of 100-200 per 100000. The incidence of ulcerative colitis is estimated to be between 9-12 per 100000, with a prevalence of 205-240 per 100000^[2]. IBD is associated with high health care costs, and can result in a significant quality of life burden. Unlike ulcerative colitis, which is limited to the mucosa, Crohn's disease typically causes transmural inflammation, and can result in stricturing and penetrating complications. The goals of therapy are two-fold, and include in-

duction and maintenance of remission, and avoidance of complications. Remission has traditionally been defined as the achievement of clinical remission, but a more recent trend has been towards achieving mucosal healing, or deep remission^[3]. These goals are achieved through lifestyle modification, medical management, and surgery when necessary. Though the underlying etiology of these diseases remains poorly understood, it is thought that Crohn's disease and ulcerative colitis are driven by an inappropriate immune inflammatory response to gut microbes, in a genetically predisposed host^[4]. The role of immunity is reflected in the focus on immunosuppressive medications in inducing and maintaining remission.

Though historically reserved for patients failing "conventional" therapies (such as 5-ASAs, antibiotics, and in some cases, steroids), immunosuppressive therapies such as immunomodulators and biologics are being used earlier in an attempt to alter the natural history of IBD^[5]. Though corticosteroids are among the oldest and most effective therapies in IBD^[6], their side effect profile limits their appeal^[5], and maintenance of a steroid-free remission has become a key tenet of the management of IBD.

The purpose of this review is to summarize the available immunosuppressive options for the medical management of IBD.

IMMUNOMODULATOR THERAPIES

Immunomodulators include thiopurines [6-mercaptopurine (6-MP) and azathioprine (AZA)], methotrexate (MTX), and cyclosporine (CSA).

Thiopurine analogues

Crohn's disease and ulcerative colitis: Though for many years these medications have been widely used as steroid-sparing agents for the maintenance of remission in moderate-to-severe IBD, the data overall supporting their efficacy are limited and often contradictory, particularly in ulcerative colitis^[7,8]. Older data have shown that thiopurines can be particularly effective in the long-term management of peri-anal and fistulizing Crohn's disease^[9]. These drugs are not suited to the induction of remission^[7], given a mean response time of 2-3 mo^[10]. None the less, their use is widespread, and their role on the treatment pyramid well established^[1,11].

6-MP and AZA are thought to act by inhibiting lymphocyte proliferation *via* the incorporation of active drug metabolites into cellular nucleotides, which likely results in anti-inflammatory effects through suppression of T cell function and natural killer cell activity^[12,13]. AZA is the active pro-drug of 6-MP, and both are similarly converted to their therapeutic end-product, 6-thioguanine (6-TG), to the inactive metabolite, 6-thiouric acid, by xanthine oxidase, and to the hepatotoxic metabolite 6-methylmercaptopurine (6-MMP) by the thiopurine methyltransferase (TPMT) enzyme^[1,13]. Lower doses may be needed in patients with intermediate TPMT enzyme activity in order to avoid leukopenia caused by high levels

of 6-TG, and neither drug can be used at all in the 0.3% of the population deficient in the enzyme, due to the risk of life-threatening toxic complications^[10]. Xanthine oxidase inhibitors can be used to boost response in patients who preferentially shunt towards 6-MMP. Testing for TPMT and measurement of both therapeutic and toxic metabolite levels are readily available in the United States, and can be used as an adjunct to routine monitoring of blood counts and liver function tests.

Dose-independent, or hypersensitivity, reactions have been described with use of 6-MP/AZA, and include hepatitis, pneumonitis, arthritis, and fever. Perhaps the most serious dose-independent reaction is pancreatitis, which can occur in approximately 4% of treated patients^[14]. The reactions usually occur early in the course of therapy, and typically resolve with discontinuation of the medication. Minor side effects such as nausea, vomiting, and flu-like illness are possible, though thiopurines are typically well tolerated in 75% of patients using them^[15]. Serious opportunistic infections are possible, as with any immunosuppressant, but uncommon^[7,10]. With regards to the development of cancer, and in particular lymphoma, following the use of these therapies, a recent meta-analysis^[16] concluded that IBD-patients on thiopurines have a 4-fold increased risk of lymphoma, but whether this increase was due to the medication or the underlying disease could not be established. These data were comparable to those from the CESAME group^[17]. Moreover, this level of risk was well below what was deemed necessary to impart significant reduction in quality-adjusted life expectancy compared to other treatment strategies^[11,18]. Ongoing and past exposure to thiopurines has been shown to significantly increase the risk of non-melanomatous skin cancers^[19], and as such patients on these therapies should be advised to use adequate sun protection and have routine skin examinations.

There has been particular concern with regards to the association between hepatosplenic T cell lymphoma (HSTCL) and thiopurine use. HSTCL is a rare but fatal lymphoma, which appears to occur more frequently in patients with IBD as compared to the general population, though the absolute risk remains very low^[20]. The risk of HSTCL appears to be higher in patients receiving thiopurines (both for IBD or for other reasons), and especially in those with long-term exposure^[20]. A 2011 review^[20] of all cases of HSTCL in IBD identified 2 other factors, male gender and age 10-35, as being associated with the development of HSTCL. Though combination therapy with thiopurines and anti-tumor necrosis factors (TNFs), particularly in this cohort of young males with IBD, has been postulated to portend an even high risk for developing HSTCL, this has not actually been demonstrated and is more theoretical, particularly since there were no cases of IBD patients treated with anti-TNF monotherapy who developed HSTCL. None the less, the authors concluded that combination therapy should be used in patients with IBD only when a clear benefit was expected^[20]. Combination therapy is discussed in more detail below.

Methotrexate

Crohn's disease: MTX is a folic acid antagonist, and is thought to act by interrupting DNA synthesis and increasing adenosine^[21], and by inhibiting interleukin (IL)-1 and suppressing T cell function. Its role in the management of IBD is much less well established than that of thiopurines. There is evidence to support the use of parenteral MTX in induction and maintenance of remission in refractory, steroid-dependent Crohn's disease^[8]. There are no convincing data to support its use in ulcerative colitis - the few studies that exist are limited both by size, quality, and the fact they used lower doses than what was shown to be effective in Crohn's disease (15 mg/wk *vs* 25 mg/wk)^[8].

MTX is thought to be safe and tolerable. Nausea can occur in 15% of patients, but can typically be prevented with the co-administration of folate 1 mg/d. Leukopenia, hepatotoxicity, hypersensitivity pneumonitis, and opportunistic infections have been reported but are uncommon. MTX is teratogenic and should never be used in pregnant women or those contemplating pregnancy^[8,13].

Cyclosporine

Ulcerative colitis: CSA is a calcineurin inhibitor, and is thought to act by decreasing pro-inflammatory lymphokines by inhibiting their antigen-induced secretion through the binding of calcium calmodulin-dependent protein phosphatase calcineurin^[22].

The data with respect to the use of calcineurin inhibitors in IBD are very limited. CSA has proven to have promise in the induction of remission of refractory ulcerative colitis, but the data with regards to its use in Crohn's disease are less convincing. Oral cyclosporine has not consistently been shown to be effective in the induction of remission of Crohn's disease (though one study showed a modest effect at higher doses)^[23-25]. Uncontrolled data demonstrated some effectiveness in treating fistulizing Crohn's disease with parenteral CSA^[26]. Studies in patients with refractory ulcerative colitis have shown that when used in the acute setting, as a bridge to thiopurine maintenance, CSA can be valuable in inducing remission and delaying or avoiding colectomy^[27].

CSA is generally considered to be less safe than other IBD therapies, because of the risk of serious side effects, such as anaphylaxis, seizure, pneumocystis carinii pneumonia, and permanent nephrotoxicity^[10]. Moreover, ease of use is limited by the need for close monitoring of drug levels due the narrow gap between the therapeutic and toxic ranges^[27]. As such, CSA is typically reserved as a rescue agent for severe, refractory disease.

BIOLOGICS

Perhaps the most significant advance in the treatment of IBD has been the introduction of anti-TNF-alpha monoclonal antibodies, and subsequent biologic therapies, both for Crohn's disease and ulcerative colitis.

Infliximab

Infliximab (IFX) is a chimeric, monoclonal antibody

(75% human, 25% mouse) that targets and binds to TNF-alpha, a potent pro-inflammatory cytokine thought to play a role in gut inflammation^[28].

Crohn's disease: IFX was initially released in 1998 for the treatment of moderate-to-severe and fistulizing Crohn's disease, after it was shown to induce remission in a small, uncontrolled study of steroid-refractory patients^[28]. These findings were later replicated in a larger, placebo-controlled study^[29], where IFX was shown to induce remission in one third of steroid-refractory patients with luminal Crohn's. The landmark study that guides our current use of IFX as an induction and maintenance medication in luminal Crohn's disease is known as ACCENT I, which showed that the 58% of patients considered to be responders to an initial IFX infusion were more likely to have a sustained remission after 1 year when maintained on q8 week infusions after an initial loading period^[30]. Subsequent research has shown a benefit of long-term therapy at 5-years^[31].

IFX has also been shown to be effective in treating fistulizing Crohn's disease, resulting in both complete closure of draining abdominal and perianal fistulae at 3 mo in 55% of patients receiving IFX 5 mg/kg (compared to 13% of patients receiving placebo)^[32], and in the long-term maintenance of remission of fistulizing Crohn's disease in 36% of patients (compared to 19% in the placebo group) at week 54 follow-up (ACCENT II)^[33]. A recent small-scale retrospective study has shown IFX in combination with antibiotics to be safe and effective in treating phlegmons^[34].

Ulcerative colitis: IFX has also been shown to have benefit in the treatment of ulcerative colitis, specifically when refractory to conventional therapies. The ACT-1 and ACT-2 trials^[35] have shown that patients with moderate-to-severe active ulcerative colitis refractory to conventional treatment were more likely to have clinical response at weeks 8, 30, and 54 in the IFX group compared to placebo, and are less likely to have undergone colectomy by week 54^[36]. Though some have argued for the earlier implementation of IFX therapy in less severe (*i.e.* moderate) ulcerative colitis^[37], the use of IFX in ulcerative colitis has typically been reserved as third-line or rescue therapy.

Safety and tolerability: IFX is generally considered to be safe and tolerable, however a risk benefit analysis should be undertaken when considering its use given the potential for serious complications. Using the ACCENT I trial data^[30] as a fairly typical profile, 32% of patients were found to have had an infection requiring treatment by week 54, including 1 case of tuberculosis and 2 deaths from sepsis (out of 2863 treated patients). Development of a lupus-like syndrome was described though very rare, though the development of ANA and anti-ds DNA antibodies more common (up to 56% and 34% respectively). Antibodies to infliximab were detected in 14% of patients, and infusion reactions were much more common

in this group (16% *vs* 4%-6%). Infusion reactions were typically mild, and required discontinuation of drug in less than 1% of cases. Concomitant use of steroids and immunomodulators decreased the risk of infusion reaction. There was a 1% rate of development of malignancy, including lymphoma and non-melanomatous skin cancers. It is worth noting that data from the TREAT registry^[38], a prospective analysis looking at the safety of IFX and other medications for Crohn's disease, taking into account confounding factors such as disease severity and use of other medications, showed that the risk of serious infection and death was no greater in patients using IFX *vs* immunomodulators, and the overall incidence comparable to that among all patients with Crohn's disease. In fact, the only independent risk factor for serious infection and death that emerged was the use of prednisone, and for serious infection alone was narcotics. Similarly though there does appear to be an increased risk of lymphoma among patients with IBD using IFX, this risk has not been quantified, and the role of confounding factors not fully understood^[11].

Adalimumab

Adalimumab (ADA) is a fully humanized, recombinant monoclonal antibody against anti-TNF- α , and thus immunogenicity and the formation of antibodies is theoretically lower. Unlike IFX, ADA is administered subcutaneously. The safety profile of ADA is similar to that of IFX, which was discussed previously.

Crohn's disease: ADA has been shown to be effective in inducing^[39] and maintaining^[40] remission in IFX-naïve patients with moderate-to-severe Crohn's disease, those with loss of response to IFX^[41], and in patients with fistulizing Crohn's disease^[42]. Initial response rates are comparable between ADA and IFX - approximately one-third of naïve patients will achieve remission^[30,39]. The effects with regards to healing fistulas were more robust for IFX^[32,42]. Rates of mucosal healing in Crohn's disease were comparable with ADA and IFX^[43]. Rates of antibody formation were significantly less compared to IFX^[30,39].

Ulcerative colitis: ADA has also been shown to induce^[44] and maintain^[29] remission in patients with moderate-to-severe ulcerative colitis who have been refractory to conventional therapy with steroids, immunomodulators, or anti-TNFs, though the efficacy of ADA was lower in those who were not anti-TNF naïve.

Certolizuman pegol

Crohn's disease: Certolizuman pegol (CZP) is a humanized pegylated Fab fragment of an anti-TNF- α antibody. Because it does not contain an Fc portion like other monoclonal antibodies (such as IFX and ADA), CZP does not induce antibody-dependent cellular cytotoxicity. It is administered subcutaneously. It is only approved for the treatment of moderate-to-severe Crohn's disease, in

patients with inadequate response to conventional therapies, and only readily available in the United States, Russia, and Switzerland. It has been shown to have a modest improvement in response and remission rates in moderate-to-severe Crohn's disease as compared to placebo^[45,46], as well as with regards to fistulizing disease^[47]. Rates of antibody development were comparable to those with IFX^[30]. Since the lack of an Fc portion prevents the active transport of CZP across the placenta, there has been some preference for its use in women of childbearing age, however timing of administration of ADA and IFX in the third trimester can be manipulated to reduce drug concentrations in the newborn^[48]. It is generally advocated that biologic agents not be changed solely for this reason.

Natalizumab

Crohn's disease: Natalizumab (NZA) is a selective adhesion-molecule inhibitor. It is a humanized monoclonal IgG4 antibody against α -4-integrins, which are selectively involved in leukocyte transfer across the gut and brain. It has been approved for the treatment of moderate-to-severe Crohn's disease in patients who have been refractory to conventional therapies. Though initial studies looking at NZA in Crohn's disease were less promising^[49], more recent, albeit smaller studies have suggested that it may be effective in induction and maintenance of remission in moderate-to-severe Crohn's disease^[50], and in particular in patients who have lost response to anti-TNF therapies^[51].

Use of NZA has been limited due to its association with progressive multifocal leukoencephalopathy (PML), a devastating demyelinating CNS infection caused by the reactivation of JC virus^[52]. Because of this, it can only be prescribed in the United States through a restricted distribution program. JC virus antibody testing is available, but its use controversial as a screening tool in patients at high risk of developing PML. The majority of normal individuals are seropositive for JC virus, and any immunosuppressed patients are at risk for *de novo* infection. Using JC viuria as a marker for latent infection with high risk of reactivation is promising, but more research is needed^[53].

Promising therapies

Golimumab: Golimumab is a fully human anti-TNF therapy, administered subcutaneously. It was recently approved in the United States for treatment of patients with refractory ulcerative colitis based on phase 2 and phase 3 studies showing efficacy over placebo in induction and maintenance of remission. Further studies are needed before use of this medication becomes more widespread^[54,55].

Ustekinumab: Ustekinumab is a fully human IgG1k monoclonal antibody that blocks biologic activity of IL-12 and IL-23, an inflammatory pathway thought to be linked to the pathogenesis of Crohn's disease^[56]. A phase 2b clinical trial was recently published, and demonstrated improved rates of induction response to therapy among primary and secondary anti-TNF non-responders

compared to placebo, however failed to demonstrate significant improvements over placebo in actual induction of remission^[56]. None the less, phase 3 data have not yet been published, and this drug remains promising.

Vedolizumab: Vedolizumab in an investigational, humanized monoclonal antibody that selectively inhibits migration of lymphocytes into the gut by exclusively targeting alpha-4-beta-7 integrin. By being more highly selective than other anti-integrin therapies, specifically NTZ, vedolizumab is not thought to carry the same risk of PML, though long-term data are extremely limited^[57]. Though phase 2 data demonstrated a positive trend, vedolizumab was not shown to induce clinical response in Crohn's disease^[58]. Results were much more favourable in ulcerative colitis, where vedolizumab was found to be superior to placebo in inducing and maintaining remission^[59], however more studies are needed.

Combination therapy

Therapy with anti-TNF-alpha antibodies and other biologics is limited by loss of efficacy and antibody formation to the drug, underscoring the need for further research and development of novel therapies. Concomitant use of immunomodulators has been shown to decrease antibody formation and boost longevity of biologic medications. The landmark SONIC trial^[60] compared efficacy and safety of IFX and ADA alone *vs* in combination for Crohn's disease. The primary end-point of corticosteroid-free remission at week 26 was achieved by approximately 56% of patients in the combination group, *vs* 44% and 30% in the IFX and AZA groups, respectively, and this significant difference persisted through week 50. There were also significantly higher rates of mucosal healing in the combination group, without any significant increase in infections.

With respect to ulcerative colitis, the UC SUCCESS trial data, available only in abstract form to date, demonstrated superiority of IFX and AZA compared to monotherapy with either agent in inducing remission, but did not show benefit of combination therapy over IFX alone in achieving mucosal healing. This cohort was only followed for 8 wk, so no conclusion can be drawn with respect to maintenance of remission^[61].

CONCLUSION

Though the underlying genetic and molecular pathways responsible for the development and severity of IBD remain poorly understood, the therapeutic focus, particularly for more advanced disease, has been on immunosuppressive medications. The goal of therapy remains maintenance of a steroid-free remission, though striving for a deep remission with mucosal healing is becoming more standard. Advances in the understanding of the molecular basis of Crohn's and ulcerative colitis have led to the development of promising new biologic therapies, which will likely be studied further both as monothera-

peutic agents, and for use in combination with immunomodulators.

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WJG 20th Anniversary Special Issues (3): Inflammatory bowel disease

Management of intestinal failure in inflammatory bowel disease: Small intestinal transplantation or home parenteral nutrition?

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Core tip: In this review we describe and compare the principal options for the management of intestinal failure in patients with inflammatory bowel disease: home parenteral nutrition and intestinal transplantation. We describe patient survival, complications and quality of life considerations that influence individualised decision-making between approaches. As survival from transplantation improves, decision-making is likely to change.

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Abstract

Inflammatory bowel disease and Crohn's disease in particular, is a common cause of intestinal failure. Current therapeutic options include home parenteral nutrition and intestinal transplantation. For most patients, home intravenous therapy including parenteral nutrition, with a good probability of long-term survival, is the favoured choice. However, in selected patients, with specific features that may shorten survival or complicate home parenteral nutrition, intestinal transplantation presents a viable alternative. We present survival, complications, quality of life and economic considerations that currently influence individualised decision-making between home parenteral nutrition and intestinal transplantation.

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INTRODUCTION

Intestinal failure (IF) may result from obstruction, dysmotility, surgical resection, congenital defect or disease-associated loss of absorption^[1]. It is characterized by the inability to maintain protein-energy, fluid, electrolyte and/or micronutrient balance^[1]. Three categories exist: types 1, 2 and 3^[2]. Type 1 generally occurs post-operatively and is self-limiting [such as a patient developing an ileus, requiring short-term parenteral nutritional (PN) support for days or even weeks]. Type 2 most commonly develops in individuals with sepsis following major intestinal resection. Patients require nutritional support for many weeks

or months, pending definitive surgery that may reverse dependency on PN. Type 3 is irreversible, for which long-term home parenteral nutrition (HPN) is required and is the focus of this article.

Intestinal failure in inflammatory bowel disease

Crohn's disease (CD) is most commonly associated with type 3 IF, but the overall incidence is low. The point prevalence of type 3 IF as a percentage of all causes in the United Kingdom in ulcerative colitis (UC) is 3% and 29% in CD^[3]. UC is much less commonly associated with type 3 IF because the small intestine is uninvolved, although IF can still occur through complications arising from delayed colectomy in immunocompromised patients, early re-operation, or mesenteric infarction after colectomy.

When IF does occur in patients with CD, it is usually due to one of three reasons: as a result of complications of surgery for intra-abdominal sepsis, extensive primary small bowel disease impairing nutrient absorption, or uncomplicated sequential resection leading to a shortened small bowel. The first is the principal cause of IF in CD^[4]. Following a first small bowel resection, the reported risk of IF in patients with CD at 5, 10, 15 and 20 years is 0.8%, 3.6%, 6.1% and 8.5% respectively^[5]. Predisposing factors to type 3 IF in CD include younger age at diagnosis and (at first operation) stricturing disease or family history of inflammatory bowel disease^[6]. In addition, the CD susceptibility gene nucleotide-binding oligomerization domain-containing protein 2 (NOD2) is associated with IF in patients without CD^[7]. Whether this also applies to CD remains to be proven, despite established associations between NOD2 mutation and small bowel CD^[8].

MANAGEMENT OPTIONS FOR INTESTINAL FAILURE

Three options exist for the management of patients with type 3 IF: HPN, intestinal transplantation (ITx) and intestinal lengthening.

Home parenteral nutrition

HPN has formed the standard of care for managing patients with type 3 IF for several decades^[9,10]. Early regimes were complicated, but solutions have evolved to mixed-nutrient, stable, "single" or "bipartite" bags, meeting a patient's tailored nutritional requirements^[9,11]. These solutions can be delivered through long-term percutaneous intravenous catheters, specialised pumps and patients specially trained in self-administration, or specifically trained nursing staff. CD is the principal indication for HPN in the United Kingdom, although other disease aetiologies, such as cancer, form the principal indication in other countries^[12-15].

Intestinal transplantation

The first human small bowel transplant was in 1964

but, like many early organ transplants, the graft failed to survive^[16,17]. It was only after refinements in immunosuppression that ITx started to show promise, with the first successful small bowel (+ liver) transplant taking place in 1988, enabling the recipient to achieve nutritional autonomy^[18]. With further advances in immunosuppression and operative techniques, the number of transplants performed annually rose until 2005, since when it has remained stable^[19]. In the United Kingdom alone, the number of intestinal transplants has increased from single figures (2000-2008) to 14-22/year (2011-2013)^[20]. Currently, 100 ITxs are performed per year in adults, primarily in North America and Europe; 65% of ITx transplants between 2006 and 2011 were indicated for short bowel syndrome (SBS), of which 13% were in patients with CD^[19]. The number of transplants performed annually in other parts of the world, such as Asia and South America, is much lower, but gradually increasing^[19]. In total, 20 ITxs were reported to have been performed in Japan between 1996-2010, while Australia and India have both recently reported their first cases^[21-23]. To the best of our knowledge, there are no published reports of ITx in Africa.

At present, three types of graft transplants are performed: isolated intestinal, combined liver-intestinal and multivisceral transplantation. Combined liver-intestinal grafts include intestine, duodenum, liver and pancreas. Multivisceral grafts include intestine, stomach, duodenum, pancreas, possibly liver and colon, or other organs. At present, isolated small intestinal transplants are the commonest, although abdominal wall transplantation is increasingly combined with intestinal transplantation and provides a readily accessible marker for rejection^[19,24].

Currently, the choice between HPN and ITx as primary therapeutic options for patients with type 3 IF is principally driven by predicted survival outcome. Thus, HPN, with its superior long-term survival, remains the first-line management option for most patients with type 3 IF, with ITx being reserved primarily for those with HPN-associated complications and/or high risk of death from their underlying disease (Table 1). However, if transplant experience and survival continues to improve, other factors, such as patients' quality of life (QoL), will enter decision-making when balancing HPN against ITx.

Intestinal lengthening

Intestinal lengthening procedures involve the lengthways division of a dilated small intestine and subsequent end-to-end anastomosis ("Bianchi procedure") or sequential zig-zag stapling of a dilated small intestine (serial transverse enteroplasty or "STEP procedure")^[25-28]. Lengthening techniques were pioneered in children with SBS, and have rarely been performed in adults, although recent European and American experience suggests they may be a viable treatment option for SBS. However, as only one series has reported their use in 2 patients with CD, lengthening procedures will not be discussed further in this review^[29]. Instead, this review will focus on the choice between HPN and ITx in patients with type 3 IF.

Table 1 Intestinal transplantation indications^[53,57,92,94]

North American	European
Indications Failure of home parenteral nutrition (HPN) Impending or overt liver failure Central venous thrombosis of ≥ 2 central veins Frequent and severe central venous catheter-related sepsis Frequent episodes of severe dehydration despite intravenous fluids in addition to HPN High risk of death attributable to the underlying disease Intra-abdominal invasive desmoids tumour Congenital mucosal disorders Ultra-short bowel syndrome Intestinal failure with high morbidity and low acceptance of HPN Need for frequent hospitalisation, narcotic addiction or inability to function Patient's unwillingness to accept long-term HPN	Indication Irreversible, benign, chronic intestinal failure with no possibility of bowel rehabilitation associated with life threatening complications of HPN Individual case-by-case decision for all patients Non-indications High risk of death due to underlying disease Chronic dehydration Significantly impaired quality of life

PATIENT SURVIVAL

Survival on home parenteral nutrition

HPN series provide survival information, although series (Table 2) excluding cancer as a primary disease indication clearly have more relevance to IBD and ITx^[14,30-32]. Of the latter, one series (1986-2001; $n = 40$) reported 1-, 3- and 5 year probability survivals on HPN of 97%, 82% and 67%^[31]. Another study (1990-2006; $n = 268$) excluding malignancy, but only including patients with SBS, reported 1-, 5- and 10-year actuarial HPN survivals of 94%, 70% and 52%^[30]. However, as 46% regained nutritional independence, most within 1 year, and only 6% had CD, this study does not represent ITx candidates or most patients with CD needing intravenous nutritional support. Indeed another study (1979-2003; $n = 188$), including 7% with malignancy (4% active neoplasia; 3% desmoid), showed that patients with CD on HPN ($n = 60$) had a better 5-year probability survival than all other patients (87% *vs* 77%)^[33]. This is further supported by a review of case series, which reported the 10-year survival rate for patients with CD to be 88% in comparison to 62% for SBS due to other causes and 60% for pseudo-obstruction^[34]. Thus, in general, patients with CD have the best probability of surviving long-term on HPN, which may reflect their age or limited co-morbidity (compared, for example, with those with SBS from mesenteric infarction). However, not all patients with CD have similar chances of long-term survival, as generic series show

that having < 50 cm of remaining small bowel (RR = 7.7) or an end-enterostomy (RR = 6.2) are associated with worse survival^[35,36].

Survival following intestinal transplantation

Survival following ITx (Table 2) currently appears worse than for patients on HPN, with the American National Registry reporting 1-, 3- and 5 year survivals of 77%, 61% and 51% for all primary adult ITxs (1987-2009; $n = 687$)^[37]. The trouble with direct comparisons with HPN survival data is, however, simple: the patient populations differ, since only a minority on HPN and predominantly those with established complications from HPN would be considered to be transplant candidates. Series highlight that graft and patient survival have improved considerably since earlier transplants; the American National Registry demonstrates a rise in 1-year survival from 69% in 1998 to 79% in 2007^[38]. This improvement is particularly evident in centres performing larger case volumes; for example, 5-year survival in Pittsburgh improved from 40% in 1990-1994 to 68% in 2001-2003^[19,39].

There are few survival data specific to adults with CD, with only one multi-centre series (1987-2009; $n = 86$) reporting 1-, 3- and 5 year survivals of 79%, 53% and 43% in patients with CD as the primary cause of IF^[24]. As in other series, 5-year survival from more recent procedures (2001-2009) has increased (62% isolated ITx; 57% liver-ITx). Post-ITx survival in adults with CD therefore appears comparable to those of other diseases. As with all patients, negative predictors for post-ITx survival in CD include age > 40 years and hospitalization prior to ITx^[24,37]. Furthermore, although the presence of NOD2 mutations are associated with an increased risk of rejection, graft loss and death in all patients post-ITx, this effect is not specific to CD^[40].

HOME PARENTERAL NUTRITION COMPLICATIONS

Catheter-related complications

Complications (Table 3) including catheter-related blood stream infections (CRBSI) and central venous thrombosis (CVT) are a significant cause of morbidity in patients requiring HPN and form part of the indications for ITx (Table 1).

Reported rates of CRBSI vary from 0.1/1000-2.41/1000 catheter days^[41-43]. Centre practices may influence rates; for example, increased use of lipid infusions or catheter use for infusions other than PN, are associated with an increase in CRBSI^[44]. CD may also increase risk, with one series of patients with CD describing 57% of patients having at least one CRBSI within the 7.9 years follow-up, and another series comparing patients with and without CD, reporting an association between CD and infections, attributed to immunosuppression and/or genetic immunodeficiency^[5,45]. Most CRBSI are bacterial (some are fungal) and remain a major concern, with between 4.5% and 16% of all HPN deaths attributed to CRBSI^[35,36,46]. It

Table 2 Comparison of patient survival

		1-yr	3-yr	5-yr	10-yr
Home parenteral nutrition	Series of 40 patients excluding malignancy (1986-2001) ^[31]	97%		82%	67%
	Series of 268 patients with SBS and excluding malignancy (1990-2006) ^[30]	94%		70%	52%
	Patients with Crohn's disease (CD) extracted from multiple series ^[34]				88%
	Series of 60 patients with CD (1979-2003) ^[33]			87%	
	Series of 453 patients (1990-2008) ^[39]	85%		61%	42%
Intestinal transplantation	Series of 687 patients (1987-2009) ^[37]	77%	61%	51%	
	Series of 86 patients with CD (1987-2009) ^[24]	79%	53%	43%	

is however clear that meticulous patient and carer training can achieve the very low CRBSI rates reported by some centres^[41,43].

Catheter-related CVT is less common than CRBSI, with recent series reporting 0.06-0.16 episodes of CVT/1000 d PN^[33,43,47]. Nevertheless, at one centre, the mean number of thrombosed central veins per patient at the point of ITx assessment, was 1.495^[48]. In an older series of patients with CD on HPN (1987-2009; *n* = 86), 50% were reported to have exhausted vascular access^[24]. This is clearly a concern for patients facing ITx, where vascular access is of paramount importance. CVT remains a prime consideration when determining an individual's referral for ITx assessment^[48,49].

Intestinal failure-associated liver disease

Intestinal failure-associated liver disease (IFALD) in children can be graded as early/mild, established/moderate and late/severe based on biochemical, histological and clinical parameters^[50]. With late disease, clinical and radiological signs of liver failure are accompanied by extensive hepatic fibrosis. IFALD incidence varies between centres, with one series reporting no patient with a bilirubin > 50, no decompensated liver disease or IFALD-related deaths in 107 HPN patients over a median of 40 mo (range: 4-252 mo)^[51]. Meanwhile, at the other extreme, another series of 90 HPN patients (median HPN duration 49 mo, range: 6-198 mo) reported complicated liver disease (as defined by bilirubin > 60, decompensation or fibrosis/cirrhosis on biopsy) in 50% of patients at 6 years; there were 6 IFALD-related deaths in the latter series^[52]. IFALD is associated with increased risk of death on HPN, but in light of its variable frequency, mortality also differs between centres (0%-22% of deaths)^[51-53]. These differences may reflect differing HPN management decisions, leading to variable exposure to risk factors, such as excess calories (especially lipids), underlying diseases (*e.g.*, bacterial overgrowth in CD) and recurrent episodes of sep-

Table 3 Potential complications of home parenteral nutrition and intestinal transplantation

Home parenteral nutrition	Intestinal transplantation
Catheter-related blood stream infection	Allograft rejection
Catheter-related central venous thrombosis	Infection
Intestinal failure-associated liver disease	Graft <i>vs</i> host disease
	Post-transplant lymphoproliferative disease
	Renal failure
	Disease recurrence

sis^[51,52,54,55]. Careful PN lipid formulation certainly seems to have a role in prevention and treatment^[51,52,56].

Given its association with death, IFALD is an indication for ITx in most countries^[53,57]. Patients with impending (raised bilirubin, progressive thrombocytopenia, or splenomegaly) or overt liver failure (portal hypertension, hepatosplenomegaly, fibrosis, or cirrhosis) should be considered for ITx^[53,57]. Traditionally, stratification of waiting times for liver-ITx was influenced by the model for end-stage liver disease (MELD), and paediatric version, pediatric end-stage liver disease. However, deaths on the waiting list in those awaiting combined liver-ITx were 8 times higher compared to liver alone^[38]. As a result these scores were adjusted to incorporate a sliding scale of 10% mortality at 3 mo. Over time this has reduced time waiting for a transplant, increased the number of liver-ITx and narrowed the gap between the two groups in both paediatric and adult populations^[58]. In addition, the MELD score and C-reactive protein have been shown to be independent predictors of survival in IF and may also be considered as reasons for early ITx assessment^[59]. Future areas for research include algorithms that may predict risk of developing IFALD. In reality, whilst liver biopsy remains the gold standard for assessing hepatic disease, non-invasive markers, such as Fibroscan[®], are gathering popularity. Rigorous data on its predictive value are needed. If a Fibroscan[®] score equated to a level of hepatic injury that in turn predicted the risk of IFALD, then there would be a strong argument for tailoring lipid exposure and total caloric intake to reduce this risk. As yet there is insufficient evidence to justify its use as a monitoring tool for patients on HPN.

Assessment tools

The Cambridge-Miami (CaMi) assessment tool has undergone preliminary validation to predict ITx outcome according to an individual's venous access and co-morbidity^[48,49]. It was developed as a pre-operative scoring system to help quantify the likelihood of survival after isolated ITx or as a composite graft, to help assess patients. The score combines risk factors for early-, medium-, and long-term survival, including loss of venous access and impairment of organs or systems not corrected by transplantation, each scored 0-3. Initial validation examined the preoperative scores of 20 patients who had

received intestinal transplants either isolated or as part of a cluster graft, who had either been followed up postoperatively for at least 10 years, or died within 10 years and compared with their survivals. A CaMi score < 3 was associated with survival ≥ 3 years (12/12 patients) and > 3 with survival < 6 mo (4/4). It is simple, disease-specific and is undergoing prospective validation, but does not examine QoL.

INTESTINAL TRANSPLANTATION COMPLICATIONS

Post-ITx complications (Table 3) may result in graft failure or death. Graft failure leads to patients resuming HPN and the need to consider re-transplantation, which has a lower probability of success than the index transplant^[37]. Graft failure is common, with reasons including allograft rejection, graft-*vs* host disease (GVHD), infection, post-transplant lymphoproliferative disorder (PTLD), primary non-function, or technical complications^[39]. Most graft failure occurs within the first few years. The North American Registry reported graft failure rates at 0.5-, 1-, 3- and 5 years of 16%, 26%, 46% and 48%^[60]. Graft survival in CD (2001-2009; $n = 63$) at 1-, 3- and 5 years is reported to be 90%, 65% and 52% for isolated-intestinal grafts and 65%, 57% and 57% for liver-intestinal grafts^[24]. Notably, the reason why liver-ITx grafts in the latter series of CD patients fared worse than in patients with other primary disease remains unexplored.

Allograft rejection

Rejection occurs *via* an immune-mediated response, which may be acute (cellular or vascular) or chronic^[39]. Although the incidence of rejection has fallen with improvements to immunosuppressive regimes, it remains a common problem. While not all episodes of rejection result in graft loss, they are associated with substantial morbidity^[39]. Acute cellular rejection has been reported to occur in 50%-75% intestinal transplants (1990-2008; $n = 500$) varying, with immunosuppressive regime, while acute vascular rejection occurred in 6% of isolated intestinal grafts (1990-2008; $n = 215$), of which 92% responded to treatment with anti-lymphocyte therapy^[39]. Chronic rejection occurred in 15% of all grafts (1990-2008; $n = 500$), but as indicated above, liver-containing grafts showed a significantly better chance of avoiding rejection than liver-free grafts, presumably due to the transplanted liver's immune-protective properties^[39,61]. In patients with CD, acute rejection has been reported to be the commonest cause of graft failure in the first 3 mo (33%), while chronic rejection was the commonest cause between 1-5 years (28%)^[24].

Infection

Immunosuppression minimises rejection, but renders recipients vulnerable to environmental and donor infections, with resultant morbidity and mortality^[62]. Infec-

tions are the second commonest cause of graft failure, accounting for 11% failures in a general ITx series (1990-2008) and 18% (1987-2009) in a CD ITx series^[24,39].

In one study 100 infections were reported in 19 ITx recipients during a median 524 d (18 mo) follow-up, with 94% having at least one bacterial infection^[63]. A larger study (1994-2001; $n = 124$) reported 2.6 episodes/patient^[64]. Bacterial infections are commonest, representing 61% of infections in one series, with septicemia in 15%^[64]. However, the risk of fatal bacterial infections has declined following changes to immunosuppression regimes^[39].

Viral infections, particularly cytomegalovirus (CMV) and Epstein Barr virus (EBV), are potent causes of post-ITx morbidity, but the risk is declining, with altered immunosuppression regimes, viral monitoring and prophylaxis, and the matching of CMV donor to recipient status^[39,65,66]. In a recent series, (2001-2008; $n = 322$) 11% of ITx recipients were infected but none died^[39].

Graft vs host disease

ITx recipients are at high risk of developing GVHD, with one centre (1994-2007; $n = 241$) reporting GVHD in 9% of recipients, with children being at greatest risk (12.4% *vs* 4.6% adults, $P = 0.05$)^[67]. Isolated ITx have a lower risk than multivisceral grafts (4.4% *vs* 13.2%, $P = 0.05$). When GVHD does occur, it has a high mortality: in one series (1990-2008; $n = 500$), 18% of those affected died^[39]. There are no data to show whether ITx recipients with CD as the primary disease have an altered incidence of GVHD.

Post-transplant lymphoproliferative disease

Immunosuppression increases the risk of malignancy (8.7 times higher than general population), with the commonest being PTLD, which is associated with 1% of graft failures (2001-2008) and a high mortality (29% affected died; 1990-1995)^[39,68,69]. Recipients may be affected early or late following ITx, as shown by rates of 2.5%, 5.3%, 7.2%, 8.2% and 10.2% at 0.5-, 1-, 2-, 3- and 5 years post-ITx in one series (2005-2009)^[60]. Risk factors include EBV infection, which is present in 97%, immunosuppression and splenectomy^[39]. CD has not been investigated as a risk factor for PTLD.

Renal failure

Renal dysfunction is common in patients requiring HPN due to chronic dehydration from SBS and oxalate nephropathy, associated with jejuno-colonic anastomoses that are not uncommonly formed following CD resection. Although recurrent episodes of dehydration may be considered an indication for ITx, the actuarial incidence of significant renal dysfunction as a referral criterion for ITx (usually including multivisceral transplant) is uncommon^[53].

The risk of chronic renal failure is higher following ITx than in patients remaining on HPN^[70]. In the first year following ITx, 80% of adults experience an episode of acute kidney injury^[71]. Isolated small intestinal reci-

ents have a significant decline in renal function at 1 year, but multivisceral recipients do not, which may relate to their differing immunosuppressive regimes, since high dose tacrolimus is a risk factor^[71-73]. At one centre, 9% of surviving adult recipients required renal replacement therapy during a median follow-up of 7.6 years, with 50% attending for dialysis and 50% undergoing renal transplant^[74]. Furthermore, renal dysfunction at 1 year is a risk factor for mortality^[72]. Whether or not patients with CD undergoing ITx have an increased risk of renal impairment due to oxalate exposure or other factors remains unexplored.

Disease recurrence

Patients may view ITx as a cure for CD and, theoretically, donor graft genetics may reduce the risk of CD recurrence. However, case reports describe 2 patients, transplanted in 1994, who later developed clinical and histological recurrence (7 mo and 8 years post-ITx)^[75,76]. In another series, up to 19% of ITx survivors with initial CD had a recurrence suggested on routine histological assessment, but this did not affect graft function^[74]. Similarly, another small study reported asymptomatic CD recurrence in 50% (2/4) of patients, which was evident only on mucosal biopsy specimens (granulomatous enteritis)^[77]. Patients should therefore be advised that CD may reoccur in the grafted tissue, but that this may not manifest clinically, perhaps due to the effects of post-ITx immunosuppression.

QUALITY OF LIFE

Generic and disease-orientated tools exist for the assessment of QoL. Generic tools completed by patients on HPN and/or following ITx include the SF-36, Karnofsky performance score and QoL Inventory^[74,78-80]. The value of generic tools, including EQ5D (EuroQoL) which is used by National Institute of Clinical Excellence to calculate quality-adjusted life years, is that they are validated in many diseases, allowing comparisons with QoL in other chronic conditions, and in many languages^[81]. Their disadvantage is that they give little weight to disease-specific factors, such as a stoma or need for parenteral fluids. Disease-orientated tools have been developed, including both the Short Bowel Syndrome-Quality of Life Scale for patients with SBS and the HPN-QoL, for patients with IF on HPN^[82,83], which has been partially validated. An adapted version of the HPN-QoL has been used post-ITx^[84].

Quality of life on home parenteral nutrition vs intestinal transplantation

The SF-36 and an adapted version of HPN-QoL have been used to compare patients on HPN and following ITx. One study using the adapted HPN-QoL, found ITx recipients scored statistically better for ability to holiday/travel, fatigue, gastrointestinal symptoms, stoma management/bowel movements and global health status/quality of life and non-significantly better for eating ability^[84].

However, ITx recipients scored worse for sleeping pattern. Another study using SF-36, compared ITx recipients with patients stable on HPN and those with complicated IF on HPN, who were defined as those referred for ITx but who remained on HPN for whatever reason. Better QoL in ITx recipients and patients stable on HPN was reported than in those with complicated IF on HPN, suggesting that the benefit of ITx over HPN is limited to selected patients^[85]. This is to be expected, since patients on stable HPN not being considered for ITx cannot reasonably be compared to ITx. Another study, limited by low numbers from a single centre in the comparator group, compared QoL in those transplanted with those on stable HPN and found no difference between pre-ITx and stable HPN, but a significantly higher QoL score post-ITx^[78]. Since all these studies were small ($n = 55, 22$ and 59) and included patients who had undergone a variety of grafts for differing indications and at varying intervals, larger prospective assessments with disease specific tools are needed to confirm these findings, before QoL can be used to guide ITx decision-making. The optimal study would compare outcomes of those undergoing ITx for HPN failure compared to those with poor QoL at risk of HPN failure^[78]. No studies have examined QoL pre- and post-ITx in patients with IBD.

ECONOMIC CONSIDERATIONS

Both HPN and ITx impose financial burdens on the healthcare system and the patient. HPN cost estimates differ between countries and health services. In North America, HPN is estimated to cost \$64000/year^[86]. In the United Kingdom, HPN costs £30-40000/year, for 5 d/wk if self-caring, or £55-65000/year if requiring nursing support^[87]. ITx in the United Kingdom is estimated to cost £80000 in the first year, followed by £5000 annually. Thus, assuming no complications arise, ITx should be cost-effective after 2 years^[88]. Another European group drew similar conclusions when they reported an initial HPN fee of €9006, followed by €63000 annually, compared to €73000 initially for ITx followed by €13000 annually^[89].

HPN and ITx both affect an individual's economic situation. In some countries, patients are liable for a proportion of their healthcare cost, which places pressure on the patient to be in gainful employment. Assessment of employment status has been studied, but heterogeneity of the studies has produced variable data. For example, a recent review of QoL found that the employment rate after commencing HPN was 0%-52%^[90]. In contrast, in the last 500 transplants from Pittsburgh, 31% of their 151 adult patients were in employment or education^[39]. At a subsequent paper assessing long-term outcomes, of their surviving adult patients, 41 (35%) were in employment^[74]. The only comparative study between HPN and ITx was a cross-sectional study, where demographic data in a QoL study reported 56% (6% unemployed) of ITx recipients in part or full-time employment, compared to 30% (52%

unemployed) of patients on HPN ($P = 0.013$)^[84].

INDICATIONS FOR INTESTINAL TRANSPLANTATION

Decisions regarding the role of ITx *vs* HPN in type 3 IF necessarily consider many factors. Guidelines produced by the American Society of Transplantation (AST) (Table 1) are based on the premise that HPN still offers patients the best chance of long-term survival. Current guidelines therefore state that ITx should only be considered for patients with complications associated with HPN^[57]. These vary, ranging from life-threatening IFALD, recurrent CRBSI or limited venous access from CVT. Notwithstanding limited evidence of benefit, QoL can be included in the decision-making process. Thus, of these indications, HPN failure is the commonest (62%), followed by risk of death from underlying disease (26%) and high morbidity IF or low acceptance of HPN (12%)^[91]. More recent European guidelines suggest that indications for ITx should be restricted to complications associated with a higher mortality and do not support ITx for indications such as chronic dehydration or poor QoL^[92]. Further to evaluate the indications, Pironi and colleagues recently carried out a multi-centre, 5-year prospective follow-up of 545 European patients with type 3 IF, stable on HPN; patients were divided into two groups based on their candidacy for ITx according to AST criteria (Table 1). Within these groups, only those with desmoids or IFALD were associated with an increased risk of death on HPN, leading the authors to suggest that early referral for ITx should be mandatory for patients with these conditions. By contrast, patients with central venous catheter (CVC) complications or ultra-short bowel did not have an increased risk of death on HPN. Since there was no difference in survival in these groups whether they were transplanted or not, the authors concluded that CVC complications and ultra-short bowel be considered indications for ITx on a case-by-case basis. Notably, no patient who was considered to be an ITx candidate as a result of poor QoL or chronic dehydration actually died whilst remaining on HPN. The authors therefore concluded that these complications should not be considered an indication for ITx. Relatively few patients of the entire cohort underwent a transplant ($n = 22$), with a 5-year mortality rate of 54%. All deaths in transplanted patients were related to the transplant itself or to complications resulting from immunosuppression.

After this paper the European Society of Parenteral and Enteral Nutrition suggested that – at least in Europe – indications for ITx should be restricted (summarised in Table 1). This conclusion has been questioned by North American colleagues among others, who highlighted that the relatively poor survival rate of transplanted European patients, compared to 75% 5-year survival in a larger ($n = 182$) North American series over the same period^[93]. Indeed, it has been suggested that the poor European survival may relate to inadequate experience, since data

from the International Intestinal Transplant registry demonstrate improved graft survival in centres performing more cases^[19,93]. This trans-Atlantic debate remains unresolved, with Pironi and colleagues pointing out that some European ITx candidates (catheter complications and ultra-short bowel) had comparable survival figures on HPN and post-ITx to those of equivalent Pittsburgh ITx recipients^[93]. A key point when considering the risks and benefits of ITx *vs* HPN is that while ITx centre experience and/or outcomes may vary, the same is equally true of HPN experience and outcome. As earlier indicated, quality HPN outcomes such as the incidence of IFALD and catheter-related complications, vary appreciably between different HPN centres^[34,36,42,51,52]. Consequently, while ITx survival is likely to continue to improve and indications for ITx will shift as experience evolves, it is also essential that patients with type 3 IF are managed in expert IF centres with optimal HPN quality outcomes^[53].

CONCLUSION

Current management options for patients with irreversible IF secondary to IBD are HPN and ITx. For most patients, HPN has the more favourable survival and complication profile, but for selected patients, such as those with IFALD or specific catheter-related complications, ITx may offer better survival. As experience and outcomes in ITx improve, indications for ITx will no doubt widen. In the meantime, further work into tailoring the indications for ITx to individual patients will facilitate better selection. Since patients with CD have one of the better outcomes on HPN, the future use of tools such as CaMi, along with tailoring selection based on the predicted survival on HPN according to the primary disease aetiology, will facilitate patients' choice between ITx and HPN.

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Microscopic features of colorectal neoplasia in inflammatory bowel diseases

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Abstract

The risk of developing dysplasia leading to colorectal cancer (CRC) is increased in both ulcerative colitis and Crohn's disease. The prognosis of CRC may be poorer in patients with inflammatory bowel disease (IBD) than in those without IBD. Most CRCs, in general, develop from a dysplastic precursor lesion. The interpretation by the pathologist of the biopsy will guide decision making in clinical practice: colonoscopic surveillance or surgical management. This review summarizes features of dysplasia (or intraepithelial neoplasia) with macroscopic and microscopic characteristics. From an endoscopic (gross) point of view, dysplasia may be classified as flat or elevated (raised); from a histological point of view, dysplasia is separated into 3 distinct categories: negative for dysplasia, indefinite for dysplasia, and positive for dysplasia with low- or high-grade dysplasia. The morphologic criteria for dysplasia are based on a

combination of cytologic (nuclear and cytoplasmic) and architectural aberrations of the crypt epithelium. Immunohistochemical and molecular markers for dysplasia are reviewed and may help with dysplasia diagnosis, although diagnosis is essentially based on morphological criteria. The clinical, epidemiologic, and pathologic characteristics of IBD-related cancers are, in many aspects, different from those that occur sporadically in the general population. Herein, we summarize macroscopic and microscopic features of IBD-related colorectal carcinoma.

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Key words: Inflammatory bowel disease; Dysplasia; Colorectal cancer; Microscopic features

Core tip: The risk of developing dysplasia leading to colorectal cancer is increased in both ulcerative colitis and Crohn's disease. The biopsy interpretations will guide decision making in clinical practice: colonoscopic surveillance or surgical management. This review summarizes histological features of dysplasia and colorectal cancer in inflammatory bowel disease.

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INTRODUCTION

The most common types of inflammatory bowel disease (IBD) are ulcerative colitis (UC) and Crohn's disease (CD). The risk of colorectal cancer is increased in both UC^[1]

and CD^[2,3]. The prognosis of colorectal cancer (CRC) may be poorer in patients with IBD than in those without IBD^[4]. It is the pathologist's biopsy interpretations that guide the management of patients during surveillance^[5]. Pathologic interpretation of specimens for evaluation of dysplasia constitutes a critical step in endoscopic surveillance programs or surgery. Ultimately, it is the pathologist's interpretation of mucosal biopsy specimens that distinguishes high-risk from low-risk populations and triggers recommendations for either continued surveillance or surgery. Thus, an accurate diagnosis of dysplasia (or intraepithelial neoplasia) is the most important step in the surveillance process.

We review here the pathological characteristics of IBD-related colorectal cancer and dysplasia.

Dysplasia (intraepithelial neoplasia) in IBD

Most CRCs, in general, develop from a dysplastic precursor lesion. Patients with IBD develop dysplastic lesions that can be polypoid, flat, localized, or multifocal^[6]. Colorectal dysplasia may be defined as an unequivocal neoplastic alteration of the intestinal epithelium that remains restricted within the basement membrane within which it originated^[7]. It is synonymous with the term *intraepithelial neoplasia* adopted by the World Health Organization and Vienna nomenclature systems (Table 1) for gastrointestinal neoplasia^[5].

ULCERATIVE COLITIS

Macroscopic classification of dysplasia

From an endoscopic (gross) point of view, dysplasia may be classified as flat or elevated (raised)^[8-11]. Flat dysplasia refers to endoscopically undetectable lesions, whereas raised dysplasia refers to any type of endoscopically detectable lesion^[12].

Raised dysplasia: Endoscopically visible dysplastic raised lesions within an area affected by UC can be divided in adenoma-like and non-adenoma-like lesions on the basis of their macroscopic characteristics^[12]. Raised lesions with dysplasia in UC have been broadly separated into those that appear similar to non-IBD related sporadic adenomas, referred to as “adenoma like” and those which do not resemble adenomas: “non-adenoma-like (the former term “DALM”)^[13]. Adenoma-like RLDs represent well circumscribed, smooth or papillary, non-necrotic, sessile, or pedunculated polyps that are usually amenable to removal by routine endoscopic methods^[13,14]. Non-adenoma-like lesions include velvety patches, plaques, irregular bumps and nodules, wart-like thickenings, stricturing lesions, and broad-based masses^[9,15-17] and are not usually amenable to removal by colonoscopic polypectomy. Non-adenoma, and adenoma like RLDs are differentiated on the basis of their gross (endoscopic) appearance. Histologic comparisons of individual morphologic features in DALMs and adenoma-like dysplastic polyps have indicated that DALMs show increased ar-

Table 1 Vienna classification of gastrointestinal epithelial neoplasia

Category	
1	Negative for neoplasia/ dysplasia
2	Indefinite for neoplasia/ dysplasia
3	Non-invasive low-grade neoplasia (low-grade adenoma/ dysplasia)
4	Non-invasive high-grade neoplasia High-grade adenoma/ dysplasia Non-invasive carcinoma (carcinoma <i>in situ</i>) ¹ Suspicion of invasive carcinoma
5	Invasive neoplasia Intramucosal carcinoma ² Submucosal carcinoma or beyond

Reproduced from Schlemper *et al*^[94]. ¹Non-invasive indicates absence of evident invasion; ²Intramucosal indicates invasion into the lamina propria or muscularis mucosae.

chitectural disarray^[18], villous architecture, and inflammation^[19], but these criteria have not been evaluated longitudinally and have so far lacked the statistical power to guide the management of patients with raised dysplasia^[10,18,19].

Dysplastic polyps that are either encountered in non-diseased areas of the colorectum, for example, proximal to the transition zone in UC^[20], or have a non dysplastic pedicle^[19,21,22], are considered to be sporadic adenomas unrelated to the colitis and are managed accordingly. The endoscopic, histologic, and prognostic similarities between adenoma-like polyps in IBD and sporadic adenomatous polyps suggest that some, if not all, of the former are merely fortuitous adenomas, a conclusion that is also supported by limited molecular-based evidence^[23]. Follow-up studies after their endoscopic removal have reported no significant excess risk for the development of CRC^[24-28]. This favorable outlook is maintained even when the resected polyps contain HGD^[28,29]. Adenoma-like lesions can be adequately treated by polypectomy provided the lesion can be completely excised, shows the absence of dysplasia at the margins of the specimen, and there is no evidence of flat dysplasia elsewhere in the colon, either adjacent to, or distant from, the raised lesion^[12].

Kisiel *et al*^[30] showed that, while polypectomy may be safe for the management of adenomas occurring in most UC patients, the 5-years cumulative incidence of a combined endpoint (cancer or flat dysplasia) was 13%. Such patients should be followed closely.

Flat dysplasia: Flat dysplasia refers to dysplasia that is detected unexpectedly in random biopsies of mucosa without a corresponding macroscopic lesion, although occult dysplasia is a more suitable term considering that small or subtle raised lesions might easily go unnoticed in the inflammatory background of IBD^[31]. Retrospective endoscopic studies have suggested that most dysplastic lesions are in fact endoscopically visible. On the basis of

Table 2 Biopsy classification of dysplasia in inflammatory bowel disease

Negative for dysplasia	Positive for dysplasia
Normal mucosa	Low-grade dysplasia
Inactive (quiescent) colitis	High-grade dysplasia
Active colitis	

a review of random and targeted surveillance biopsies in 525 subjects with UC during a period of 15 years, Rutter *et al*^[9] reported that 85 of 110 (77.3%) biopsy specimens of dysplasia or cancer corresponded to macroscopically visible lesions, whereas 25 (22.7%) were invisible. Similarly, on the basis of a review of surveillance biopsies in 46 subjects with UC during a period of 10 years, Rubin *et al*^[32] reported that 38 of 65 dysplastic lesions (58.5%) and 8 of 10 cancers (80.0%) were visible as 23 polyps and masses, 1 stricture, and 22 mucosal irregularities. Only some of the dysplastic lesions described as flat by endoscopists correspond to expanded mucosa resembling diminutive adenomatous polyps, whereas most correspond to histologically flat mucosa in which the crypts have been colonized by dysplastic epithelium, without alteration of the overall mucosal architecture.

Microscopic classification

Currently, dysplasia is separated into 3 distinct categories: negative for dysplasia, indefinite for dysplasia, and positive for dysplasia (low or high grade) (Table 2)^[7]. While endeavouring to minimize disagreement in both terminology and interpretation, rates of agreement using this grading system are only fair among both expert and community pathologists^[33]. Crude rates of agreement among experts have ranged from 42% to 72%; kappa values, where there is a correction for chance agreement, have remained fair for both experts and community pathologists^[33-36]. Unfortunately, rates of agreement are lowest for the indefinite for dysplasia and low-grade dysplasia categories^[24,33]. Based on these data, the CCFA consensus guidelines and the United States Multisociety Task Force strongly recommend that a second examination of the biopsies should be performed by an independent pathology expert prior to definitive treatment^[21,37].

The morphologic criteria for dysplasia are based on a combination of cytologic (nuclear and cytoplasmic) and architectural aberrations of the crypt epithelium^[7,19,38]. Cytologic features that pathologists use to evaluate the presence or absence and degree of dysplasia include the nuclear/cytoplasmic (N/C) ratio of the cells; loss of cell polarity; an increase in the number and location of mitoses (typical and atypical); the degree of nuclear stratification within the epithelium; the degree of chromasia of the nuclei (an increase is referred to as “hyperchromasia”); the presence, size, and multiplicity of nucleoli; the size and regularity (or lack thereof) of the contour of nuclei; and the variation in the size and shape of nuclei between different cells (nuclear pleomorphism). Cytoplasmic characteristics include the degree of mucinous depletion;

the number, location, and shape (normal or dystrophic) of goblet cells; and the presence or absence of surface maturation, which is defined as the progressive acquisition of cytoplasmic mucin, a decrease in the size of nuclei, and the degree of stratification of the cells, from the crypt base to the mucosal surface. Architectural features that are important for the determination of dysplasia include villiform change of the epithelium and the presence or absence and degree of crypt budding, branching, and crowding; the latter is referred to commonly as a “back-to-back” glandular growth pattern. In addition, the contour of the crypts, the degree of irregularity, and the presence or absence of intraluminal bridges (“cribriforming”) are important architectural features that are used to evaluate dysplasia in IBD^[39].

Negative for dysplasia: “Negative for dysplasia” applies to epithelium that is regenerative in nature. In the presence of active inflammation, cryptitis, crypt abscesses, or ulceration, all of which are common in the active phase of IBD, the epithelium can undergo marked reactive changes that, in some circumstances, may mimic some of the “atypical” features of dysplasia. In general, nondysplastic (“reactive”) epithelium in IBD exhibits only mild or moderate cytologic atypia coupled with preservation of crypt architecture; however, a significant degree of atypia may be present in markedly reactive epithelium adjacent to ulcerated mucosa, an area in which the architecture of the crypts may be altered as well. One of the hallmarks of reactive crypts is a base-to-surface epithelial maturation gradient in which phenotypically immature, mitotically active, basal colonocytes differentiate into mature surface cells, featuring small, normochromatic nuclei, distinct absorptive and goblet cell phenotypes, and absent mitoses^[5]. Pathologists need to exercise caution when evaluating dysplasia in ulcerated mucosa, and these areas should be avoided by the endoscopist when obtaining mucosal biopsies. Given the subtle gradation of changes, the progressive acquisition of molecular mutations that occurs in the progression of dysplasia in IBD^[38,40,41], and the wide range of morphologic patterns of atypia that is related to epithelial regeneration and repair, regenerating epithelium, particularly in the setting of active inflammation or ulceration, may reveal a level of atypia that occasionally is difficult to distinguish from true dysplasia^[41]. In these situations, pathologists use the “indefinite for dysplasia” diagnostic category. In reality, this diagnostic category is used most often as a result of one of the following circumstances: the presence of technical (tangential sectioning) or staining issues that makes interpretation of cytologic or architectural features difficult, atypia related to inflammation or ulceration, or for the rare instances in which dysplasia-like changes are present only in the crypt bases. Naturally, the frequency of the use of this diagnostic category is directly proportional to the “comfort” level and experience of the reviewing pathologist, and is one of the reasons why it is highly recommended to confirm any potential diagnosis of dysplasia with at least

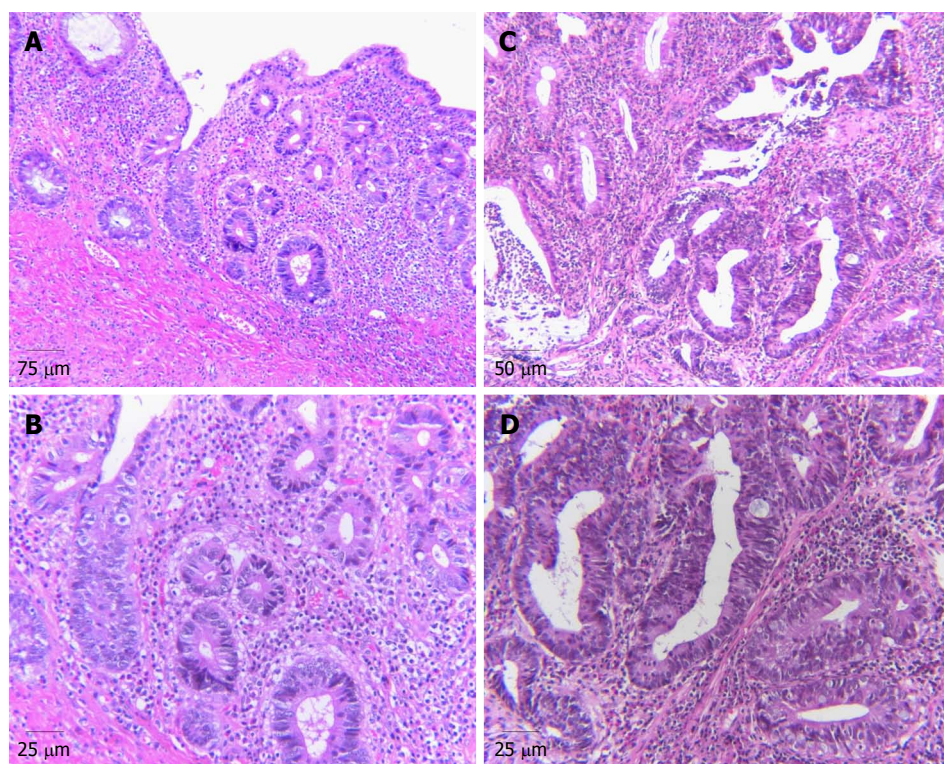


Figure 1 Microscopic features of dysplasia in ulcerative colitis. A: A minor degree of architectural aberration may occur in low-grade dysplasia (HE stain, $\times 100$); B: Low-grade dysplasia is characterized by epithelium that contains cells with significant nuclear hyperchromaticity, enlargement, and elongation. The cytoplasm is mucin depleted, and, as a result, is hypereosinophilic (HE stain, $\times 200$); C: The degree of architectural aberration is more prominent in high-grade dysplasia. Architectural aberrations, such as a complex crypt branching, or a back-to-back growth pattern that is characterized by dysplastic crypts that show little or no intervening lamina propria, also may be present (HE stain, $\times 400$); D: Full-thickness nuclear stratification, significant loss of cell polarity, nuclear pleomorphism are characteristic features of high-grade dysplasia (HE stain, $\times 400$).

one other experienced IBD pathologist before definitive treatment^[21,38].

Low-grade dysplasia: Low-grade dysplasia is characterized by epithelium that contains cells with significant nuclear hyperchromaticity, enlargement, and elongation; the last is referred to as “pencil-shaped” or “adenomatous” nuclei. Nuclei in low-grade dysplasia often show a clumped chromatin pattern, multiple nucleoli, or a single large nucleolus. Typically, the cytoplasm is mucin depleted, and, as a result, is hypereosinophilic. A decrease in the number of goblet cells and unusually oriented goblet cells, referred to as “dystrophic” goblet cells, may also be observed (Figure 1B). Dysplastic cells are usually organized in a stratified manner, but in general, the nuclei are limited to the basal half of the cell cytoplasm, without full thickness stratification. Mitotic figures may be prominent, but there are only usually a few atypical mitotic figures. Most importantly, dysplastic epithelium usually does not show surface maturation, except in rare circumstances. A minor degree of architectural aberration may occur in low-grade dysplasia (Figure 1A), but significant architectural aberration is normally diagnostic of high-grade dysplasia (Figure 1C)^[39].

High-grade dysplasia: With progression to high-grade dysplasia, the degree of cytologic or architectural aberration

is more prominent (Figure 1C). Cytologically, full-thickness nuclear stratification, significant loss of cell polarity, nuclear pleomorphism, and an increase in the number of normal appearing and atypical mitoses, often at the level of the surface epithelium, are characteristic features of high-grade dysplasia (Figure 1D). In some instances, high-grade nuclei are more round or oval in contour, and also show a higher N/C ratio. Architectural aberrations, such as a complex crypt budding or branching, or a back-to-back growth pattern that is characterized by dysplastic crypts that show little or no intervening lamina propria, may also be present (Figure 1C). Cystic change, villiform surface change, and cribriforming are also features of high-grade dysplasia^[42-44].

Immunohistochemical or molecular markers of dysplasia: Many studies have been published in an effort to help identify sensitive and specific immunohistochemical or molecular markers that may aid in the differentiation of dysplastic from reactive epithelium in IBD. p53 and Ki67 have been studied the most extensively. Most of the markers that were evaluated previously have been linked, in some capacity, to the development of cancer, and include those involved in control of cell proliferation (*e.g.*, Ki67, cyclin D1), intercellular adhesion (β -catenin, e-cadherin), DNA content, mucin or glycoprotein histochemistry, and tumor suppression (p53)^[40,45-59].

The *p53* gene shows an increase in the frequency of mutations in the dysplasia-carcinoma progression in IBD^[45-51]. *p53* is a common early mutation in the dysplasia-carcinoma sequence in IBD, and, as a result, many investigators have evaluated the role of *p53* in helping to differentiate reactive from dysplastic epithelium. For instance, in a study by Wong *et al.*^[45], a moderate degree of *p53* staining was detected in almost 50% of reactive cases, but strong *p53* staining was seen only in cases of true dysplasia. Unfortunately, although *p53* expression increases progressively from low- to high-grade dysplasia and carcinoma, some studies showed that the epithelium that is considered indefinite, or even negative, for dysplasia, may be *p53* positive; this diminishes its usefulness as a marker of true dysplasia^[42,45,47]. Furthermore, *p53* overexpression can be detected in a small proportion of cases that are considered morphologically negative for dysplasia^[45,47,49,50]. In addition, several studies in other tissues have revealed a high rate of false-positive staining in the absence of *p53* mutations, and a high frequency of false-negative staining as well^[60,61]. Nonspecific binding of *p53* to non-*p53* mutation-related antigens may also lead to false-positive results. Furthermore, *p53* results may vary substantially depending on the specific type of antibody used. For instance, some *p53* mutations result in the production of a protein that does not bind to some antibodies that are directed against the wild-type protein. Finally, there is no known antibody, or combination of antibodies, in use that can detect all *p53* mutations^[60]. For these reasons, *p53* immunostaining is not routinely used but can be helpful in rare cases to differentiate reactive from dysplastic epithelium in IBD.

Several studies showed that dysplasia expresses markers of cell proliferation at higher levels in the crypt, and in the surface epithelium, compared with biopsies that are considered negative for dysplasia^[45,46,58]. Unfortunately, there is much overlap between reactive epithelium and dysplasia in this regard, so evaluation of cell proliferation is not useful in individual cases to distinguish these lesions.

Recently, immunostaining for alpha-methylcyl-CoA racemase (AMACR), an antibody that is often used in the assessment of diagnostically difficult atypical, and potentially neoplastic, lesions of the prostate, was shown to have a high degree of specificity for detection of dysplasia in the GI tract, such as in Barrett's esophagus and IBD^[62]. In this recent study by Dorer and Odze^[62], AMACR was not expressed in any mucosal biopsy in UC that was considered negative for dysplasia; however, it was increased significantly in foci of low-grade dysplasia (96%), high-grade dysplasia (80%), and adenocarcinoma (71%) with a specificity for neoplasia of 100%. Thus, AMACR is a new, potentially useful immunohistochemical marker that pathologists may use in their arsenal when trying to differentiate reactive from dysplastic epithelium in IBD. More recently, Chen *et al.*^[63] showed that Chitinase 3-like-1 may contribute to the proliferation, migration and neoplastic progression of colonic epithelial cells

under inflammatory conditions and could be a useful biomarker for neoplastic changes in patients with IBD. More recently, Ludwig *et al.*^[64] show that PDCD4 nuclear expression may be usefully applied as ancillary marker in the histological assessment of IBD-associated dysplastic lesions.

Overall, dysplasia diagnosis is essentially based on morphological criteria.

Crohn disease

Less studied than in UC, dysplasia in CD occurs more often in areas close to, rather than distant from, the primary tumor mass. Dysplasia in CD is often multifocal^[65]. In a study by Sigel *et al.*^[66], dysplasia was found adjacent to carcinoma in 87% of cases and distant from carcinoma in 41% of cases. Microscopic features that are used for a diagnosis of dysplasia (or intraepithelial neoplasia) in CD are the same that those used in UC dysplasia.

Colorectal carcinoma in inflammatory bowel disease

The clinical, epidemiologic, and pathologic characteristics of IBD-related cancers are, in many aspects, different from those that occur sporadically in the general population. For instance, cancers that occur in IBD, and particularly UC, tend to be distributed more evenly throughout the length of colon, are more likely to be multiple in number and tend to be of higher histologic grade than with sporadic carcinomas^[67]. In some studies, up to 27% of IBD-related cancers are multiple in number^[68,69]. In addition, there is a higher prevalence of mucinous carcinomas in IBD^[67,70,71]. More recently, there has been a shift to a higher incidence of early-stage tumors (stage I - II) compared with IBD-related cancers from previous decades^[41,67,68]. Contemporary studies show that 50%-60% of newly diagnosed IBD-related cancers are stages I or II. Of course, this may be due to a combination of many factors, such as an increased level of awareness and early detection by colonoscopic surveillance. In one study, by Delaunoy *et al.*^[67], of 290 patients who had IBD (241 with UC and 49 with CD) and an equal number of age- and sex-matched patients who had sporadic colorectal cancer, UC-related carcinomas were diagnosed at a younger age and tended to be distributed more evenly in the colon, compared with sporadic tumors.

Macroscopic features

Pathologically, IBD-related tumors often grow in a more diffuse fashion than sporadic cancers, and may be more difficult to detect grossly because they may be raised only minimally above the level of the surrounding mucosa^[41,67]. The gross appearance of cancers in IBD is heterogeneous. They may be strictured, ulcerated, irregular, polypoid (pedunculated or sessile), or nodular or they may appear as an irregular plaque or bump^[41,71]. Some tumors may be entirely microscopic, without any grossly evident mucosal abnormality^[17,72]. A disproportionately higher percentage of cancers in IBD, including UC, occurs in strictured segments of colon^[73,74].

Microscopic features

Microscopically, most IBD-related carcinomas are adenocarcinomas. Mucinous carcinomas make up a high proportion, up to 50% in some studies^[40]. In addition, signet ring cell adenocarcinomas are 10 times more common in IBD than in the general population^[41,75]. Rarely, IBD-related adenocarcinomas may be extremely well differentiated and consist of widely separated, regularly arranged, bland-appearing glandular profiles that contain only mildly atypical unilayered neoplastic epithelium with low-grade cytologic atypia, and without desmoplasia^[41,76]. These tumors may arise from mucosa that shows little or no definite evidence of dysplasia. Other types of carcinomas, such as neuroendocrine carcinomas, mixed adenocarcinoma/squamous cell carcinoma, undifferentiated carcinoma, and even pure squamous cell carcinoma (particularly in the distal rectum and anal canal in CD), have been encountered in IBD; some occur with increased frequency^[40,41,73-83]. However, these tumors are rare and are often reported as single case reports or as small series.

Colorectal carcinoma in CD

Regarding CD cancers specifically, recent evidence suggests that the risk of cancer in CD is similar to that in UC, particularly for patients who have long-standing and extensive colonic disease^[38,40,84,85]. In contrast to UC, CD patients who develop cancer are often older in age, but are much younger than patients who develop sporadic colon cancer in the general population^[41,68,86]. Some early reports cited a higher left-sided predominance for cancers in CD compared with UC; however, several contemporary studies showed a more equal distribution of tumors in the colon in CD, similar to UC^[73,67,87]. Earlier studies may have been biased by the fact that cancers in CD often occur in and around the anal canal related to fissures or fistulas^[86-88]. As a result, there is an increase in the incidence of pure squamous cell carcinomas in CD compared with UC. Although most cancers in CD are believed to occur within inflamed portions of intestine^[40,89,90], in some studies, up to 42% of patients who had CD developed tumors in areas of mucosa devoid of endoscopic or pathologic evidence of inflammation^[73,86-88,91]; however, this may be due to treatment effect^[92]. Finally, surgically excluded segments of colon or small bowel also are considered particularly prone to the development of carcinoma in CD, but this is postulated to be related to the fact that excluded segments of inflamed bowel remain at risk for carcinogenesis for longer periods of time over the course of the patient's life^[40,41,67,87,93]. Because surgical procedures that result in preservation of inflamed but excluded segments of bowel are performed only infrequently in patients who have CD, cancers in surgically excluded segments of bowel are now uncommon.

ing carcinoma is related to the extent of the patient's disease (pancolitis *vs* left-sided disease), duration of disease, and level of activity. In IBD there is abundant evidence to support the theory that cancer develops through an inflammation-dysplasia-carcinoma sequence. Several molecular events involved in the chronic active inflammatory process contribute to multistage progression of carcinoma development. Morphologic identification of dysplasia in IBD is the best and most reliable marker of an increased risk for malignancy. Future advances in, for example, stool DNA assays or the use of confocal endomicroscopy or endoscopic ultrasound may help in the identification of high risk patients and the assessment of dysplastic lesion^[11].

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CONCLUSION

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WJG 20th Anniversary Special Issues (3): Inflammatory bowel disease

Venous thromboembolism in patients with inflammatory bowel disease: Focus on prevention and treatment

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Abstract

Inflammatory bowel disease (IBD) patients have an increased risk of venous thromboembolism (VTE), which represents a significant cause of morbidity and mortality. The most common sites of VTE in IBD patients are the deep veins of the legs and pulmonary system, followed by the portal and mesenteric veins. However, other sites may also be involved, such as the cerebrovascular and retinal veins. The aetiology of VTE is multifactorial, including both inherited and acquired risk factors that, when simultaneously present, multiply the risk to the patient. VTE prevention involves correcting modifiable risk factors, such as disease activity, vitamin deficiency, dehydration and prolonged immobilisation. The role of mechanical and pharmacological prophylaxis against VTE using anticoagulants is also crucial. However, although guidelines recommend thromboprophylaxis for IBD patients, this method is still poorly implemented because of concerns about its safety and a lack of awareness of the magnitude of thrombotic risk in

these patients. Further efforts are required to increase the rate of pharmacological prevention of VTE in IBD patients to avoid preventable morbidity and mortality.

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Key words: Inflammatory bowel disease; Venous thromboembolism; Thromboembolic prophylaxis; Anticoagulants; Unfractionated heparin; Low molecular weight heparin

Core tip: Inflammatory bowel diseases (IBD) patients have an increased risk of venous thromboembolism (VTE) that represents a significant cause of morbidity and mortality. The prevention of VTE involves the correction of modifiable risk factors, such as: disease activity, vitamin deficiency, dehydration and prolonged immobilization. Essential is also the role of mechanical and pharmacological prophylaxis. However, thromboprophylaxis in IBD patients, although guideline-recommended is still poorly implemented because of concerns about its safety and, over all, the lack of awareness of the magnitude of thrombotic risk in these patients. Further efforts are required to increase the rate of pharmacological prevention of VTE in IBD so to avoid some preventable morbidity and mortality.

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INTRODUCTION

Inflammatory bowel diseases (IBD), including Crohn's dis-

ease (CD) and ulcerative colitis (UC), are chronic disorders that predominantly affect the bowel; however, IBD can also be associated with numerous extraintestinal complications. Among these complications, venous thromboembolism (VTE) is particularly important, due to both its high prevalence and its significant morbidity and mortality^[1-9]. However, despite extensive evidence supporting the association between IBD and VTE, among physicians, there is still a lack of recognition of this risk, with dangerous consequences for patients^[8,10]. Thus, the aim of this review is to summarise the most recent evidence regarding the prevention and treatment of VTE in IBD patients in light of the newest epidemiological data on this feared association.

EPIDEMIOLOGY AND CLINICAL FEATURES OF VTE IN IBD PATIENTS

Epidemiological data

IBD patients have a 2- to 3-fold increased risk of developing deep venous thrombosis (DVT) and pulmonary embolism (PE) compared with the general population^[1,3-10]. In their population-based cohort study, Bernstein *et al*^[7] found an incidence rate of DVT of 30.7 per 10000 person-years in IBD patients (30.0 for UC patients and 31.4 for CD patients) and 14.9 per 10000 person-years for PE in the entire IBD population (19.8 for UC and 10.3 for CD). The overall relative risk (RR) of VTE reported in this study was of 3.47 (95%CI: 2.94-4.09)^[7]. These findings were confirmed by a recent, large population-based study from Denmark reporting an incidence rate of VTE of 24 per 10000 person-years among IBD patients (24.4 for UC patients and 23.3 for CD) compared with an incidence rate of 13.4 per 10000 person-years in a non-IBD cohort matched for age and gender^[4]. Although the incidence of VTE increases with age, the highest RR for VTE was observed among patients younger than 40 years of age^[3-7], whereas no significant differences linked to sex or the type of IBD were found^[1-10]. VTE occurred more frequently during phases of active disease and in patients with extended disease (pancolitis in UC patients and extensive colonic involvement in CD)^[3-6]. Recently, Grainge *et al*^[5] conducted an epidemiological study that aimed to quantify the risk of VTE during different activity phases of IBD. The researchers confirmed that IBD patients had the highest risk of VTE at the time of a flare (hazard ratio of 8.4 compared with controls), although an increased risk still persisted during disease quiescence (hazard ratio of 2.1 compared with controls). These findings support the hypothesis of a procoagulant tendency in IBD patients^[2,11]. Indeed, Miehsler *et al*^[3] demonstrated that VTE is a specific feature of IBD because neither rheumatoid arthritis, another chronic inflammatory disease, nor celiac disease, another chronic bowel disease, was accompanied by an increased risk of VTE compared with controls. Furthermore, these data were confirmed by the finding that among 17 chronic illnesses that were evaluated using the

United Kingdom primary care General Practice Research Database, only cancer and heart failure carried a greater risk of VTE than IBD did^[12]. Interestingly, another study reported that the RR of VTE in pregnant females with IBD was even greater than in pregnant females without IBD with an OR of 8.44 (95%CI: 3.71-19.20) for UC and 6.12 (95%CI: 2.91-12.9) for CD^[13]. Regarding the mortality rate in patients with IBD and VTE, the existing data indicate, significant 2.5-fold-increased odds of mortality associated with VTE-related hospitalisations compared with non-VTE-related hospitalisations^[8]. In addition, a revisit of a series of 98 IBD patients with VTE evaluated at the Mayo Clinic over a decade (1990-2000) reported a 22% mortality rate^[6], which is similar to the 18% mortality rate that was reported in a cohort of IBD patients with VTE two decades earlier at the same institution^[9].

VTE location

VTE occurs primarily in the deep veins of the legs and in the pulmonary system and, less frequently, in the cerebrovascular system, portal vein, retinal vein, and mesenteric veins (Figure 1)^[14-17]. Recently, a cohort study aimed to determine the location and clinical features of the first VTE in IBD patients and confirmed this finding^[18]. Of 157 IBD patients with a history of VTE, 142 (90.4%) had DVT and/or PE, whereas 15 (9.6%) had cerebral, portal, mesenteric, splenic or internal jugular vein thrombosis^[18].

Risk factors for VTE

Although a detailed exploration of risk factors is beyond the scope of this review, we must remember that VTE in patients with IBD is a multifactorial event that involves both hereditary and acquired factors that can coexist, thereby multiplying the individual prothrombotic risk^[2,11,19]. The main modifiable acquired risk factors for VTE in IBD patients are reported in Table 1. Several are intuitively more frequent in IBD patients compared with the general population, such as dehydration, indwelling catheters, prolonged immobilisation, hyperhomocysteinaemia, surgical interventions, and active disease with an “inflammatory burden”. The mutual interactions between inflammation and coagulation have been extensively studied, and IBD represents a paradigmatic model for this complex interplay^[20,21]. Indeed, in IBD, several mechanisms triggered by active inflammation are involved in moving the coagulative balance towards a prothrombotic state, including (1) increased plasmatic levels of recognised risk factors for thrombosis, several of which are also considered to be acute-phase reactants, and decreased levels of natural anticoagulants; (2) reduced fibrinolytic activity; (3) endothelial abnormalities that are mainly represented by the downregulation of the anticoagulant thrombomodulin and endothelial protein C receptor, which in turn affects the conversion of protein C into its activated form; and (4) abnormalities of platelets, such as thrombocytosis and increased activation and aggregation^[11]. Concerning the inherited risk factors for VTE, the most common factors are: factor V Leiden

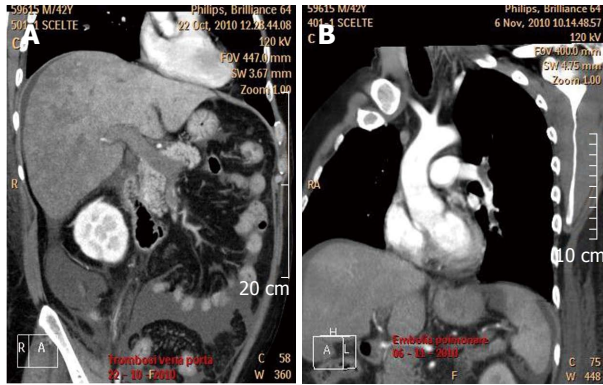


Figure 1 Computed tomography scan showing portal vein thrombosis (A) and a pulmonary embolism (B) in a patient with active ulcerative colitis.

mutation, G20210A mutation of the prothrombin gene, and homozygous C677T mutation in the methylenetetrahydrofolate reductase gene. However, no increased prevalence of these genetic prothrombotic factors has been found in IBD patients with or without VTE^[19]. Therefore, patients with IBD face both the VTE risk factors (acquired and inherited) identified in the general population and those specific to IBD, and the prevention and control of those risk factors are of paramount importance for thromboprophylaxis in this patient population.

PROPHYLAXIS AGAINST VTE IN IBD PATIENTS

Non-pharmacological prophylaxis

As described above, VTE in IBD is a multifactorial process in which acquired risk factors seem to play the most important role; therefore, these factors' prevention and/or treatment can lead to effective prophylaxis. Hydration, correction of deficiencies in vitamins (particularly in vitamins B6 and B12 and folate) that can reduce homocysteine levels^[22], graduated compression stockings or pneumatic devices, and early mobilisation after surgery should be always considered, especially in hospitalised IBD patients (Table 1). Additionally, even in the absence of direct evidence, it might be expected that the control of disease activity could decrease the risk of VTE by reducing the already-mentioned procoagulant factors that are closely associated with active inflammation. Furthermore, many drugs used for IBD treatment have shown anti-inflammatory activity and an anticoagulant effects. Indeed, mesalamine can reduce platelet activation^[23], azathioprine and 6-mercaptopurine inhibit platelet aggregation *in vitro*^[24], and infliximab normalises haemostatic parameters and reduces the amount of circulating microparticles and the levels of prothrombotic sCD40L in CD patients^[25,26].

Pharmacological prophylaxis

Prophylactic anticoagulation in IBD patients is recommended by several practice guidelines for conditions associated with a higher risk of VTE, particularly in hos-

Table 1 Acquired risk factors for venous thromboembolism in inflammatory bowel disease patients and modalities for their prevention and/or treatment

Risk factor	Prevention/treatment modality
Active disease ("inflammatory burden")	Effective anti-inflammatory treatment
Smoking	Programmes for smoking cessation
Oral contraceptive use	Advise alternative methods of contraception
Hyperhomocysteinaemia	Assess the presence of vitamin deficiency (vitamins B6 and B12 and folic acid) and correct if necessary
Dehydration	Provide adequate hydration
Prolonged immobilisation	Early mobilisation, especially after surgery; graduated compression stockings or pneumatic devices
Infections	Timely diagnosis and treatment of infections
Indwelling catheters	Limit the use of venous catheters; when possible, administer oral and enteral nutrition
Obesity	Encourage weight loss (diet, exercise)
Long-distance travel	Frequent ambulation, exercise, hydration

pitalised patients with active disease^[27-31]. Low molecular-weight heparin (LMWH) and unfractionated heparin (UH) are recommended for thromboprophylaxis in IBD patients. The recommendations for VTE prophylaxis in IBD patients that are included in the current guidelines are summarised in Table 2. Although no randomised controlled trials (RCTs) have specifically assessed the efficacy of anticoagulation for VTE prophylaxis in IBD patients, several RCTs have demonstrated that in acutely ill medical patients pharmacological prophylaxis significantly reduces the incidence of VTE^[32,33]. Only data from observational studies including IBD patients undergoing thromboprophylaxis with LMWH in the perioperative setting are available^[34,35]. Scarpa *et al.*^[34] collected data on 755 colorectal surgical procedures, 383 of which were performed in IBD patients. All patients had received 4000 IU/d LMWH from the day of operation through to the discharge. Six postoperative thromboembolic events occurred in this population, all in IBD patients; of these events, two occurred in CD patients (clinical DVT rate of 1.2%) and four occurred in UC patients (clinical DVT rate of 2.6%)^[34]. Similar data were reported by an Irish study in which the rates of postoperative VTE were evaluated both in 79 UC patients undergoing 180 major intra-abdominal surgeries and in 18 patients with familial adenomatous polyposis (FAP) undergoing 35 surgical operations of similar complexity^[35]. All patients were treated with standard perioperative VTE prophylaxis. Only three UC patients (1.7%) developed VTE, compared with no patients with FAP^[35]. Unfortunately, in both of these studies, a control group without prophylaxis was not included. Therefore, we can only hypothesise a benefit of LMWH prophylaxis because the VTE rates reported in a large cohort of hospitalised IBD patients in the United States were similar to those found in the above-mentioned studies. More specifically, Nguyen *et al.*^[8] extracting data from 73197 discharges for CD and 43645 discharges

Table 2 Published guidelines for the prevention of venous thromboembolism in inflammatory bowel disease patients

Scientific society (reference)	Recommendations	Type of population at risk
European Crohn's and Colitis Organisation (ECCO) ^[29]	Mechanical thromboprophylaxis and/or heparin administration (UH or LMWH)	UC
European Crohn's and Colitis Organisation (ECCO) ^[28]	Consider VTE prophylaxis (UH, LMWH, or fondaparinux) in all hospitalised patients	CD
British Society of Gastroenterology (BSG) ^[31]	Pharmacological VTE prophylaxis for hospitalised patients with severe UC	UC
American College of Gastroenterology (ACG) ^[30]	VTE prophylaxis with heparin for hospitalised patients with severe UC	UC
American College of Chest Physicians (ACCP) ^[27]	Mechanical thromboprophylaxis with GCS or IPC; anticoagulant thromboprophylaxis with LMWH, UH or fondaparinux when bleeding risk decreases	Acutely ill hospitalised medical patients at increased risk of thrombosis who are bleeding or at high risk of bleeding

GCS: Graduated compression stockings; IPC: Intermittent pneumatic compression; CD: Crohn's disease; UC: Ulcerative colitis; VTE: Venous thromboembolism; LMWH: Low molecular-weight heparin; UH: Unfractionated heparin.

for UC and found that the crude rates of VTE were 21 per 1000 hospitalisations for UC patients and 13.9 per 1000 hospitalisations for CD patients. However, we should note that in the population analysed by Nguyen *et al.*^[8] only 18% and 11% of CD and UC patients, respectively, underwent bowel surgery during their hospitalisations and that abdominal surgery is a strong predictor of developing VTE. In conclusion, available evidence on the efficacy of thromboprophylaxis in IBD is still scarce, and RCTs that aim to ascertain this issue are warranted.

Issues associated to the adherence to the pharmacological prophylaxis

Another key topic is the low rate of VTE pharmacologic prophylaxis in hospitalised IBD patients, and, particularly in those admitted for medical services compared with those admitted for surgery, despite the recommendations provided by the guidelines^[36,37]. The inadequate use of anticoagulants for VTE prophylaxis in IBD is mainly related to two factors: (1) gastroenterologists' lack of awareness of both the increased risk of VTE in IBD patients and the guideline-recommended use of pharmacological prophylaxis in hospitalised IBD patients^[38]; and (2) concerns about the safety of anticoagulant drugs in patients with active IBD^[36,39]. However, a recent retrospective study of 974 IBD inpatients with a reported rate of pharmacological prophylaxis of 80% at admission showed that the rates of major and minor bleeding were similar for patients who received VTE prophylaxis and those who did not^[36]. Moreover, VTE prophylaxis was not associated with major postoperative bleeding^[36]. Indirect evidence of the safety of anticoagulation in IBD patients during an active flare also comes from certain clinical trials in which UH or LMWH was used to treat UC^[40-42]. A meta-analysis of eight clinical trials showed that few serious adverse events were observed in patients treated with UH or LMWH compared with controls, with no significant difference in any trial^[41]. In particular, only in one study, three patients with moderate-to-severe UC included in the heparin group were withdrawn from the study because of worsening of rectal bleeding^[42]. One of these patients required urgent colectomy. Additionally, in

the control group, one patient developed toxic megacolon and underwent urgent surgery. The remaining seven clinical trials showed no bleeding-related adverse events in their heparin groups^[41]. All of this evidence confirms that the prophylactic use of anticoagulants in hospitalised IBD patients with acute disease is safe, despite the presence of bleeding at admission. Another unresolved issue is whether thromboprophylaxis should be extended to all ambulatory patients with disease exacerbation or only to a subgroup considered to be at a higher risk of VTE. As previously reported, the highest risk of VTE in IBD patients is during phases of active disease^[3-5]; however, in the context of active disease, Grainge *et al.*^[5] found that the RR was higher during non-hospitalised periods than during hospitalised periods (hazard ratio of 18.8 *vs* 3.2). These data suggest that hospitalisation should not be considered as the only discriminant factor for thromboprophylaxis and that anticoagulation could also be extended to a subgroup of ambulatory patients with active disease and other significant risk factors for VTE. However, this finding should be interpreted with caution because the absolute risk of VTE is much more informative than the RR, although not always known for an individual patient. In fact, in the same study, the absolute risk of VTE of a patient hospitalised for an IBD flare was nearly six times higher than the absolute risk during an ambulatory flare (37.5 per 1000 person-years *vs* 6.4 per 1000 person-years)^[5]. Thus, for each patient with active IBD, the absolute risk of VTE should be carefully assessed, including the personal and family histories of VTE, the presence of cardiovascular or respiratory diseases, obesity, information on the use of oral contraceptives and smoking status, the presence of genetic prothrombotic risk factors, reduced mobility, and the presence of venous catheters^[43]. Additionally, as previously reported, also disease features could help in assessing the individual prothrombotic risk^[3-10]. Lastly, it is well known that surgery represents a major risk factor for VTE, particularly in patients with IBD^[8], and thromboprophylaxis is universally performed during the perioperative period. A recent retrospective review of patient data obtained from the American College of Surgeons National Surgical Quality

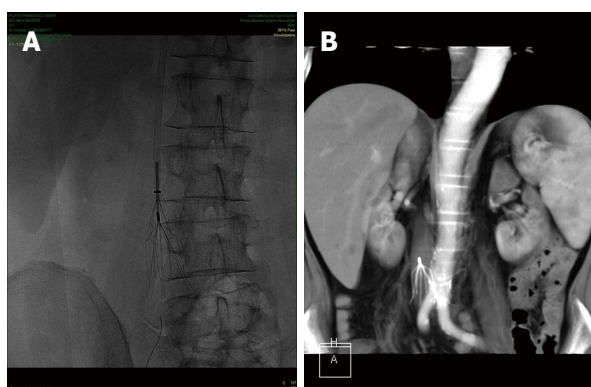


Figure 2 Plain abdominal Rx (A) and computed tomography scan (B) showing an inferior vena cava filter placed for the prevention of recurrent pulmonary embolism in a patient with Crohn's disease and deep venous thrombosis.

Improvement Program aimed to identify modifiable risk factors for short-term (30-d) postoperative VTE^[44]. The study reported that the following actions can potentially reduce the incidence of VTE in the surgical setting: correcting preoperative coagulopathy and/or anaemia, improving nutritional status, reducing steroid use, operating early to avoid emergency surgery, and limiting anaesthesia time^[44].

TREATMENT OF VTE IN IBD PATIENTS

The treatment of VTE in patients affected by IBD is the same as the treatment for subjects without IBD^[28,29]. If there is no haemodynamically significant bleeding or an indication of thrombolysis, LMWH is the ideal treatment. LMWH is usually switched to an oral vitamin K antagonist (*i.e.*, warfarin). The duration of therapy with anticoagulants is not well established because the possibility of VTE recurrence in IBD patients should be balanced with the bleeding risk caused by anticoagulant use. In fact, a recent study showed that IBD patients who experience their first episode of unprovoked VTE have a 33% risk of a second episode of VTE within 5 years, with a risk of recurrence that is 2.5-fold higher than that of non-IBD patients after an initial episode of unprovoked VTE^[45]. Nguyen and Bernstein^[46] conducted a decision analysis study to compare the costs and effectiveness of time-limited anticoagulation (for 6 mo) and extended anticoagulation for the management of VTE in IBD. The study found that among IBD patients who have had unprovoked VTE, the benefits of long-term coagulation in reducing recurrent VTE outweigh the risks of the associated bleeding. In particular, extended anticoagulation may be more appropriate for patients who developed VTE in the absence of active disease or other transient provoking factors^[46]. In the general population, local thrombolytic therapy is indicated for massive thrombosis and for life-threatening VTE, and several cases of successful catheter-directed thrombolytic treatment in IBD patients have been reported^[47]. Additionally, the placement of inferior vena cava (IVC) filters is indicated in cases of floating

thrombi in the deep veins of the legs and recurrent PE despite anticoagulant therapy and in cases with a high risk of bleeding (Figure 2)^[48].

CONCLUSION

IBD patients have a risk of VTE that is 2- to 3-fold greater than that of the general population. This risk is higher during disease flares, both for inpatients and outpatients. However, during hospitalisation, multiple prothrombotic risk factors other than active disease act synergistically, multiplying the absolute risk of VTE. Because VTE has significant morbidity and mortality, its prevention is mandatory. VTE prevention involves correcting modifiable risk factors and administering pharmacological prophylaxis. However, although guidelines recommend thromboprophylaxis for IBD patients, it is still poorly implemented because of concerns about its safety and a lack of awareness of the magnitude of thrombotic risk in these patients. Therefore, further efforts are required to increase the rate of pharmacological prevention of VTE in IBD patients to avoid preventable morbidity and mortality.

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WJG 20th Anniversary Special Issues (3): Inflammatory bowel disease

Multipotent role of platelets in inflammatory bowel diseases: A clinical approach

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Abstract

There is evidence that inflammatory bowel diseases (IBD) combine both inflammation and coagulation in their pathogenesis and clinical manifestations. Although platelets (PLT) are well known for their role in hemostasis, there are a rising number of studies supporting their considerable role as inflammatory amplifiers in chronic inflammatory conditions. IBD are associated with several alterations of PLT, including number, shape, and function, and these abnormalities are mainly attributed to the highly activated state of circulating PLT in IBD patients. When PLT activate, they increase in size, release a great variety of bio-active inflammatory and procoagulant molecules/particles, and express a variety of inflammatory receptors. These inflammatory products may represent a part of the missing link between coagulation and inflammation, and can be considered as possible IBD pathogenesis instigators. In clinical practice, thrombocytosis is associated both with disease activity and iron deficiency anemia. Controlling inflammation and iron replacement in anemic patients

usually leads to a normalization of PLT count. The aim of this review is to update the role of PLT in IBD and present recent data revealing the possible therapeutic implications of anti-PLT agents in future IBD remedies.

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Key words: Anemia; Crohn's disease; Platelets; Thrombocytosis; Ulcerative colitis

Core tip: Many platelets (PLT) changes have been described in IBD, including morphological alterations (mean PLT volume, PLT distribution width, plateletcrit, and augmented granular content), count increase, microparticles release, over-excretion of granular content, and increased formation of PLT-PLT and PLT-leukocyte aggregates, which are all linked to PLT activation induced by inflammatory agonists. In this review article, we present the multipotent role of PLT in human biological paths and emphasize on how PLT participate in the chronic intestinal inflammation process in IBD.

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INTRODUCTION

Inflammatory bowel diseases (IBD), namely Crohn's disease (CD) and ulcerative colitis (UC), are disorders that primarily affect the gastrointestinal tract. The immune system, with its active components, dominates IBD pathogenesis, but many genetic and environmental factors have been also implicated. A growing number of

studies are highlighting the importance of non-immune cells like endothelial, mesenchymal, and nerve cells, as well as platelets (PLT), as key players in the IBD inflammatory cascade^[1].

PLT dysfunction is considered as participating in IBD pathogenesis, although the existing evidence is rather weak. On the other hand, there is solid evidence supporting PLT having functions of potent proinflammatory cells in addition to their role in hemostasis. Several studies have shown that PLT constitute a crucial link between inflammation and coagulation in both UC and CD, creating a vicious circle in which participating parameters conserve and propagate each other^[2].

Many PLT changes have been described in IBD, including morphological alterations [mean PLT volume (MPV), PLT distribution width (PDW), plateletcrit (PCT), augmented granular content], count increase, microparticles (MPs) release, over excretion of granular content, and increased formation of PLT-PLT and PLT-leukocyte aggregates (PLA), which are all linked to PLT activation induced by inflammatory agonists (Table 1). In the following sections, we will present the multipotent role of PLT in human biological paths and emphasize how PLT participate in the chronic intestinal inflammation process in IBD.

PLEIOTROPIC FUNCTION OF PLT

PLT are small anuclear fragments (1-6 μm) derived from bone marrow megakaryocytes, with a 5-9 d lifespan in humans. Their primary role is hemostatic; surveying endothelial barrier consistence and interfering when vessel integrity is threatened^[3]. A significant decrease of PLT ($< 20000/\text{mm}^3$) in septic models resulted in the disruption of the endothelial barrier in clinical studies^[4]. Collagen from the exposed subendothelial layer at the injured vessel site binds to plasma von Willebrand factor and recruits circulating PLT to form a glycoprotein (GP) Ib-IX-V complex. PLT adhesion to the site of injury initiates a cascade of signaling transduction through GP VI and integrin family surface receptors. PLT become activated and transform into high affinity platforms which are suitable for participating in inflammatory reactions, ligand binding, and clot formation promotion^[5]. In addition, PLT participate in wound repair and tissue regeneration by interacting with components of extracellular matrix and endothelium^[6,7].

It has been demonstrated that PLT present innate immunological properties. They express Toll-like receptors which can bind to lipopolysaccharides on the outer membrane of gram(-) bacteria^[8]. *In vitro* and *in vivo* studies have also demonstrated that PLT can internalize pathogens resistant to clearance such as *Staphylococcus aureus* or HIV virus, promoting further PLT activation changes^[9]. Moreover, PLT stimulate the formation of extracellular DNA nets by neutrophils that trap and kill gram(-) microbes, *via* the lipopolysaccharides - Toll-like receptor 4 interaction in septic models^[10,11].

Table 1 Platelet abnormalities in inflammatory bowel disease

Number and morphological changes	Loss of discoid shape Acquisition of pseudopodia Size increase Count increase (reactive thrombocytosis) Density increase Granular content augmentation MPV value decrease PDW value increase PCT value increase
Other abnormalities	
Overproduction and excretion of granular content products	P-selectin, β -TG, PF-4, fibrinogen, vWF, fibrinolytic inhibitors, coagulation, angiogenic and mitogenic factors
Increased incorporation of receptors in PLT membrane	CD40, P-selectin, GP53, GP IIb/IIIa, receptors for chemokines, cytokines and complement components
Overproduction of PLT-derived microparticles	
PLT-PLT aggregates formation	
Increased PLT-leukocytes formation	

β -TG: β -thromboglobulin; GP: Glycoprotein; IBD: Inflammatory bowel disease; MPV: Mean platelet volume; PCT: Plateletcrit; PDW: Platelet distribution width; PF-4: Platelet factor-4; PLT: Platelets; vWF: Von Willebrand factor.

PLT can also act as mediators between innate and adaptive immune systems. When activated at inflammatory sites, they excrete large amounts of pro-inflammatory substances located in their intracellular granules^[12], by which they crosstalk, recruit, and activate leukocytes, endothelial, and immune-like cells even at distant sites. A typical example of the remote PLT actions is the ability of PLT-derived CD40 ligand (CD40L) to activate dendritic cells in the injured tissue^[13] and to stimulate immunoglobulin production by B-cell compartment^[14].

PLT ability to interact with a large variety of cells is also implicated in the generation of vascular inflammation. Endothelium dysfunction triggers PLT activation processes and possibly renders PLT as the first in line to initiate atherosclerotic immune responses. Therefore production and release of PLT highly inflammatory cargo at the injured vessel wall induces and propagates the recruitment of leukocytes and the further construction of atherosclerotic lesions.

QUANTITATIVE AND QUALITATIVE PLATELET CHANGES IN IBD

Elevation in PLT count ($> 450000 \times 10^9/\text{L}$), defined as reactive thrombocytosis (RT), may frequently occur in certain conditions like hypo- or asplenic, blood loss, acute or chronic inflammatory disorders, malignancies, and iron deficiency. The first study reporting IBD RT in 1968 by Morowitz *et al.*^[15] noted markedly-elevated concentration of circulating PLT during a period of increased clinical activity in a case series of IBD patients. This effect is the result of aberrant bone marrow throm-

bopoiesis under the influence of inflammatory mediators and the aftermath of reduced PLT lifespan due to accelerated activation and consumption of thrombocytes at the sites of inflammation.

Thrombopoiesis is mainly regulated by plasma thrombopoietin (TPO). Plasma TPO binds to C-Mpl receptors on the PLT surface, and the remaining fraction promotes thrombopoiesis by binding to the same receptors on progenitor megakaryocytes in bone marrow. Thus, in normal conditions thrombopoiesis is controlled by a negative feedback mechanism based on PLT mass in blood^[16,17]. Cytokines and other inflammatory agents, especially interleukin 6 (IL-6), promote hepatic TPO production^[18], which is considered an acute phase reactant^[19]. Heits *et al*^[20] have shown that IBD patients with thrombocytosis have elevated plasma TPO and IL-6 levels. However, the existing data are vague, as other studies display a lack of correlation between PLT number and TPO concentration, indicating other possible regulating factors in IBD RT^[21]. Although PLT count is correlated to IBD disease activity^[22], it is not considered an independent risk factor for the increased risk of thromboembolic (TE) events observed in IBD patients as it is for cancer^[23]. Properly designed and adequately powered clinical studies evaluating predictive laboratory indices for TE events in IBD are still lacking.

Moreover, some conflicting data have emerged over the last decade about the role of preoperative RT in the occurrence of chronic pouchitis in patients undergoing ileal pouch-anal anastomosis. Two studies from the Surgery Department Division of Colon and Rectal Surgery in California have pointed out that the presence of elevated PLT count before surgery was associated with an increased risk for chronic pouchitis postoperatively^[24,25], a severe complication that can result in the removal or diversion of the pouch. In discontinuity with these studies, Lian *et al*^[26] failed to predict the occurrence of inflammatory pouch disorders based on pre-colectomy laboratory tests, including PLT count. Larger prospectively well-designed series with patients requiring ileal pouch-anal anastomosis are needed in order to verify possible implication of PLT in this subject.

Chronic inflammatory disorders are connected to several morphological changes in PLT indices calculated in whole blood count, such as MPV, PDW, and PCT. The most widely-studied PLT parameter in humans is MPV. PLT volume decreases when an inflammatory process is present, which is mainly attributed to thrombopoiesis abnormalities and increased PLT consumption. Inflammatory mediators stimulate bone marrow precursors to enhance PLT generation at the cost of maturation time, delivering smaller PLT in circulation, while at the same time larger and more active PLT are consumed at inflammatory sites, as is proposed in the intestinal microvasculature of IBD patients^[27].

MPV changes are correlated to inflammatory disorders like myocardial infarction, stroke, diabetes mellitus, acute appendicitis, rheumatoid arthritis, chronic hepa-

titis B, celiac disease^[28-31], paroxysmal atrial fibrillation, obesity^[32], amyloidosis^[33], and retinal vein occlusion^[34]. Moreover, MPV could serve as a reliable predictor of high risk patients for portal venous thromboembolism^[35], acute coronary syndromes^[36], and stroke in patients with atrial fibrillation. MPV has also been proposed as a useful biomarker for early gastric, pancreatic, and hepatocellular carcinoma diagnosis^[37], dietary compliance to celiac disease and exacerbation of chronic obstructive pulmonary disease^[38].

In IBD patient studies a MPV value decrease has long been observed^[39] which has been inversely correlated with endoscopic and disease activity indices, such as C-reactive protein and erythrocyte sedimentation rate^[40-44]. This MPV reduction can be attributed to the decreased circulating reticulated PLT number that was found in patients with active UC compared to inactive and healthy control subjects^[44]. In line, studies have reported an inverse relationship between extent of intestinal inflammation and MPV in IBD patients^[40,41]. Öztürk *et al*^[45] suggested that all PLT parameters (PDW, PCT, MPV) can prove to be useful surrogate markers for IBD follow-up, as they reveal strong relationship with activity indices. We observed that MPV, PCT, and PDW were correlated with certain iron deficiency markers (soluble transferrin receptors, hemoglobin) but not with activity indices such as C-reactive protein, Crohn's disease activity index score, or simple clinical colitis activity index score in IBD patients. This observation reflects a possible role of iron capacity as a regulator of megakaryopoiesis and PLT morphology^[46]. Literature reports about MPV correlations with clinical and laboratory parameters in IBD patients are presented in Table 2.

ASSOCIATION OF PLT WITH IRON DEFICIENCY IN IBD

Anemia is the most frequent extra-intestinal manifestation of IBD, affecting approximately one third of patients^[47,48]. The most prevailing type of IBD-associated anemia is iron deficiency anemia (IDA)^[48,49]. Iron deficiency is related both with up-and downregulation in PLT count, with RT reported more frequently^[50].

Several mechanisms related to iron deficiency have been implicated in PLT overproduction. Iron scarcity could trigger an increase influx of progenitor cells to the megakaryocyte cell compartment, a diminution of PLT maturation time^[51], and the generation of high ploidy megakaryocytes. Megakaryocytes can proliferate through a procedure called endomitosis, and augment DNA ploidy and cytoplasmic volume and further abandon mitosis before cytokinesis take place^[52]. Iron deficiency may lead to the production of larger polyploid megakaryocytes capable of generating numerically more PLT, as it is observed in an iron deficient rat model^[53]. Moreover, striking amino-acid sequence homology between erythropoietin (key hormone controlling erythropoiesis) and TPO, both being members of the same hematopoietic growth

Table 2 Mean platelet volume correlations with clinical and laboratory parameters in inflammatory bowel disease patients

Ref.	Disease (n)	HC (n)	MPV correlations
Yüksel <i>et al</i> ^[41]	UC (61)	27	Reduced MPV in UC compared to HC Inverse correlation between MPV and disease activity
Järemo <i>et al</i> ^[39]	UC (18), CD (9)	12	Inverse correlation between MPV and disease extent Reduced MPV in UC patients compared to HC Inverse correlation between MPV and disease activity
Güçlü <i>et al</i> ^[42]	UC (41)	(-)	Reduced MPV in active compared to non-active disease
Voudoukis <i>et al</i> ^[46]	UC (91), CD(107)	102	Reduced MPV in IBD patients compared to HC Correlation of MPV with Hb and sTfR
Öztürk <i>et al</i> ^[45]	UC (103), CD (72)	40	Reduced MPV in IBD compared to HC MPV decreases after remission in UC MPV increases after remission in CD
Kapsoritakis <i>et al</i> ^[40]	UC (93), CD (66)	38	Correlation between MPV and disease activity indices Correlation between MPV and disease extent
Kayahan <i>et al</i> ^[44]	UC (37)	20	Correlation between MPV and disease activity indices Reduced MPV in UC compared to HC
Liu <i>et al</i> ^[43]	CD (61)	50	Reduced MPV in CD patients compared to HC MPV value did not correlate to disease activity

CD: Crohn's disease; Hb: Hemoglobin; HC: Healthy controls; IBD: Inflammatory bowel diseases; MPV: Mean platelet volume; sTfR: Soluble transferrin receptor; UC: Ulcerative colitis.

factor subfamily, could be a tempting explanation for the thrombocytosis observed in children with IDA^[54] (Figure 1). This assumption, however, is in discordance with the study by Kulnigg-Dabsch *et al*^[55] that didn't observe any alteration in PLT production with the concomitant use of erythropoietin combined to iron replacement therapy in IBD patients with RT.

A special interest in IDA associated RT has arisen over the last few years in IBD^[55,56]. In a Kulnigg-Dabsch *et al*^[55] study, iron replacement was associated with dose-dependent normalization of PLT count which remained within normal range after therapy, highlighting a regulatory rather than a toxic effect of iron on PLT. Patients presented with mildly elevated or within-normal range inflammatory indices at baseline and during treatment, demonstrating that RT could be mainly attributed to IDA rather than systemic inflammatory response^[55]. In another study, iron replacement was not only associated with PLT count decrease, but also to a significant decrease in PLT activation markers, such as P-selectin and PLT-aggregation, suggesting that iron management may express anti-thromboembolic properties in IBD patients with increased risk for TE events. However, the small number of participants and the need for study protocol modification during the active phase does not allow us to make safe conclusions^[56].

Studies have also identified a correlation between PLT count, red blood cell parameters, and anemic indices in otherwise healthy IDA patients^[57,58]. In a recent study we observed a mutual relationship between PLT count and iron deficiency parameters. Inflammatory indices (C-reactive protein, Crohn's disease activity index score, and simple clinical colitis activity index score) and iron deficiency markers (ferritin, soluble transferrin receptors, and index) were correlated to PLT count in 198 consecutive IBD patients, indicating that RT is probably a multifactorial event in which iron deficiency and inflammation

hold a major role. Moreover, taking into account the low inflammatory indices in our patients' cohort, we assumed that iron deficiency could be the main factor affecting PLT count in IBD^[46].

PLT AS ACTIVE INFLAMMATORY COMPONENTS

PLT circulate at a highly-activated state in IBD, as it is demonstrated by an increased concentration of circulating PLT activation markers in the systemic circulation of patients^[59]. This activation possibly takes place in the mesenteric microcirculation, where PLT are exposed to several inflammatory mediators^[60]. Molecules in the site of injury like subendothelial collagen, cytokines from activated leukocytes, and endothelial cells, increased local adenosine diphosphate (ADP) concentration due to reduced capillary blood flow, substances released from neighboring cells, arachidonic acid, PLT activating factor (PAF), and thrombin generation augment PLT accumulation and activation in the intestinal microvasculature in IBD^[2]. During activation, PLT lose their normal discoid shape, obtain projecting forms called pseudopodia, release an increased amount of microparticles (PDMPs), and grow in size and density. Numerous metabolic reactions happen within their cytoplasm, where various inflammatory mediators are being produced^[1,39]. Proteomic studies have identified more than 300 proteins accumulated in granules of activated PLT^[61]. PLT granules are rich in PLT factor-4, β -thromboglobulin, fibrinogen, von Willebrand factor, fibrinolytic inhibitors, coagulation V and XI factors, protein S, angiogenic and mitogenic factors (PLT-derived growth factor, transforming growth factor, endothelial growth factor, and vascular endothelial growth factor), immunoglobulins, membrane ligand proteins (P-selectin), ADP, serotonin, IL-1 β , chemokines, RANTES, IL-8, and various other substances^[12]. Certain

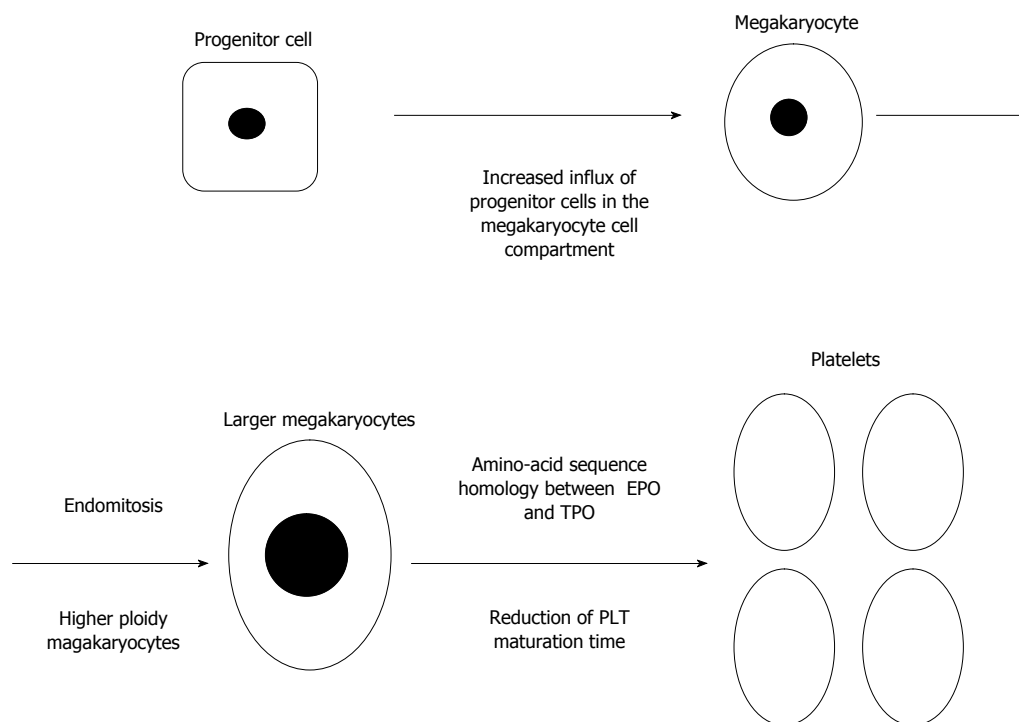


Figure 1 Possible iron deficiency mechanisms affecting platelet count in inflammatory bowel disease. PLT: Platelets; TPO: Thrombopoietin; EPO: Erythropoietin.

PLT granular products, such as P-selectin, GP IIb/IIIa, CD40L, and GP53, are incorporated into the cytoplasmic membrane, giving them a more adhesive and interacting phenotype. Moreover, PLT during activation develop receptors for chemokines, cytokines, and complement components, enabling them to participate in various inflammatory cascades in IBD^[1] (Figure 2). Molecules released from the activated PLT induce an inflammatory phenotype in endothelial cells and leukocytes. Polymorphonuclear cells enhance their superoxide, PAF, and leukotriene production, and endothelial cells stimulated by certain PLT factors (PAF, histamine, and RANTES) increase vascular permeability^[2]. CD40L(+) PLT of IBD patients induce I- and V-cell adhesion molecules (CAM) and IL-8 overexpression when co-cultured with human intestinal microvascular endothelial cells in an experimental colitis model^[62].

P selectin is a member of the CAMs family mainly produced in PLT. A soluble fraction of P-selectin is also detected in patients with inflammatory disorders, including IBD, and possibly serves as selectin binding inhibitor^[63]. The lectin containing N-terminal domain of P-selectin binds to P-selectin glycoprotein ligand (PSGL-1) found in leukocytes (mainly polymorphonuclears) mediating recruitment and rolling of infiltrating leukocytes in the gut mucosa, and initiating activation processes like chemokines production by monocytes and CD4(+) T-cells, as well as superoxide overexcretion by neutrophils^[1]. P-selectin ligation to PSGL-1 also serves in PLT-PLT aggregation and PLA formation^[2], induces tissue factor (TF) generation, and stimulates the release

of PDMPs bearing TF by leukocytes^[64]. The above mentioned findings highlight the significant role of P-selectin in IBD pathogenesis.

CD40L (CD 154) is a protein, strongly related to tumor necrosis factor (TNF) and expressed on the surface of activated PLT and immune system cells. CD40L has the ability to bind CD40 located on the surface of most immune, endothelial, and other mesenchymal cells^[65]. There are three CD40 family members encountered in humans: CD40, CD40L, and the soluble form of CD40L (sCD40L) derived by enzymatic fragmentation of CD40L in serum^[66]. The latter is believed to be produced and released only by activated PLT in IBD patients^[67]. Increased levels of CD40L(+) PLT and sCD40L are demonstrated in disorders combining inflammation and thrombosis, such as unstable angina, myocardial infarction^[68], and IBD^[67,69].

CD40L interactions have a significant role in immune mediated activation of inflammation and thrombosis. They induce TF expression by endothelial cells and monocytes^[65]. SCD40L is able to bind onto GP IIb/IIIa to promote arterial thrombosis stabilization, as was demonstrated in CD40L deficient mice^[70]. Pro-inflammatory responses of CD40L/CD40 result in chemokines, ILs, and CAMs (V-CAMs, I-CAMs, P/E-Selectin) upregulation in PLT and other immune cells^[65]. CD40L can stimulate PAF production, thus inducing PLT activation, propagating immune mediated angiogenesis in IBD in both human and murine models^[71], and provoking cytokine overexcretion by human intestinal microvascular endothelial cells such as IL-8, which constitutes a ma-

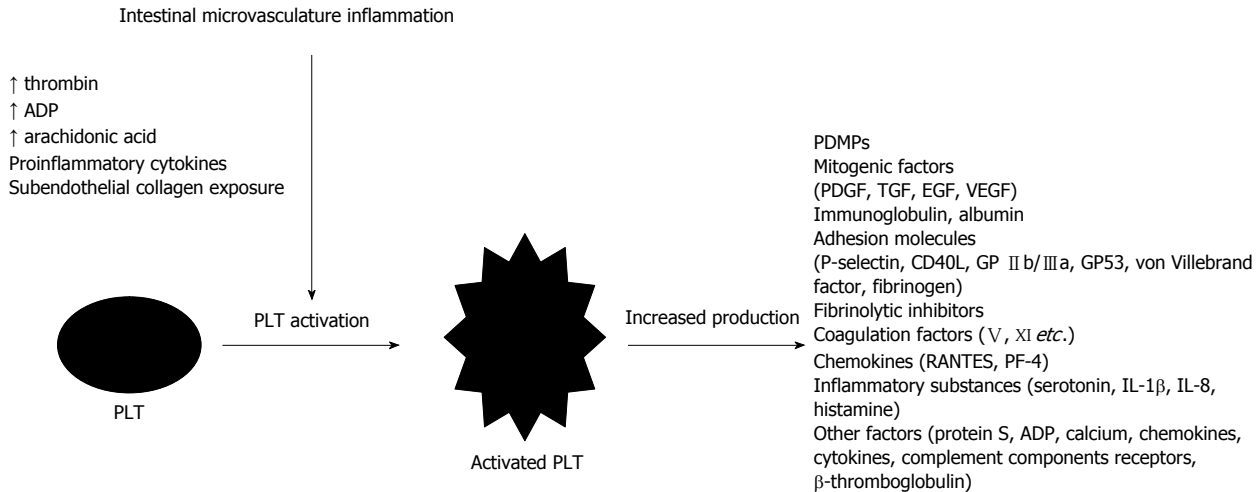


Figure 2 Factors affecting platelet function and platelet products in inflammatory bowel disease. PLT: Platelets; ADP: Adenosine diphosphate; PDMP: Platelet-derived microparticles; PDGF: Platelet-derived growth factor; EGF: Epidermal growth factor; TGF: Transforming growth factor; VEGF: Vascular endothelial growth factor; PF-4: Platelet factor-4; IL: Interleukin.

for neutrophil chemoattractant^[67]. Finally, PLT CD40L (+)-derived vesicles seem to display an immunoregulatory role by activating peripheral blood B-cells in producing immunoglobulins when co-cultured with them *in vitro*^[72] and stimulating antigen specific IgG production by germinal center modulation in the B-cell compartment^[14].

CD40L is essential in activating components of the immune system in IBD. The infiltration of neutrophils in the colonic mucosa of UC patients and macrophage chemoattraction in granulomatous lesions in CD has been found to be mediated mainly by CD40/CD40L interactions^[1]. CD40(+) immune fluorescence staining was observed only at inflamed intestinal sites and not at intact mucosal segments in intestinal endoscopic biopsies from IBD patients^[73]. A positive correlation between sCD40L and the extent of anatomical involvement in IBD was also found^[67]. Finally, CD40 deficient mice experienced significantly milder dextran sodium sulfate (DSS) colitis than wild type littermates^[74].

POTENTIAL ROLE OF PLT IN THROMBOSIS IN IBD

PLT spontaneous aggregation is a unique feature found in the blood of IBD patients that is not encountered in other inflammatory conditions^[59]. Aggregation is believed to be primarily accomplished in the mesenteric microcirculation where PLT come into close contact with increased inflammatory mediators^[60]. PLT aggregates are independent of disease activity, as their existence has been noted in colonic biopsies of IBD patients in remission, but not in healthy controls^[75].

PLT aggregation in IBD seems to represent the initial response of PLT leading to an increased risk for TE. The reported prevalence of TE events (arterial or venous thrombosis) in IBD is between 1.3% and 6.0%, with a 1.5-3.6 fold increased risk compared to the general population and other inflammatory disorders^[76,77]. The devel-

opment of TE in IBD seems to be multifactorial, with interaction of genetic and acquired factors (*e.g.*, inflammation, hospitalization, and operations). TE events in IBD indicate a higher predilection towards younger age compared to non-IBD subjects^[2]. Thromboembolism is considered a negative prognostic outcome and represents one of the four leading causes of death in these patients. Thrombosis may correlate with disease activity, but it is interesting to note that one third of the events happen during clinical remission, indicating a continuous activate state of PLT and coagulation systems in IBD^[78,79].

Moreover, the increased concentration of PLA in circulation^[80] is also considered as an aftermath of leukocyte sequestration in mesenteric circulation, where they bind to activated PLT^[81]. This interaction is mainly guided by PLT(+) P-selectin ligation to leukocytes PSGL-1. After this initial step, further ligation of PLT GP II b/IIIa to MAC-1 leukocyte membrane receptors, with fibrinogen serving as the bridging connector, intensifies binding and promotes PLA formation. PLA are major inflammatory agent carriers, more active than circulating leukocytes or activated PLT alone^[82,83] and exhibiting an enhanced ability to adhere to mucosal endothelium^[82]. Increased PLA formation is noted in many chronic inflammatory disorders like diabetes mellitus, cardiovascular and collagenous tissue diseases, asthma, systemic lupus, and rheumatoid arthritis^[84]. Therefore, PLA is indexed as a sensitive marker of inflammation and PLT activation though not in consistency with IBD activity in recent studies^[82].

PLATELET DERIVED MICROPARTICLES AND IBD

Eukaryotic cells are capable of budding small vesicles like exosomes (endosomal products), apoptotic bodies (byproducts of cell death), and MPs. Circulating MPs are a heterogeneous mixture of cellular membrane fragments that are derived from a great variety of cells, and

recapitulate the functions of their cellular origin. They influence a diverse series of physiological and pathological functions, as they can transfer genetic material (m-RNA, micro-RNA, DNA), membrane receptors, and a series of parental molecules to target cells^[85]. MP formation is a well regulated process consisting of local concentration changes in specific intracellular molecules, cytoskeleton disruption, and phosphatidylserine inversion in the outer membrane layer of ancestral cells^[86].

Although MPs are detected in low concentrations in health, a great variety of cardiovascular diseases, inflammatory disorders, cancer, and diabetes are associated with increased MP production. They are considered major procoagulant factors, due to TF and phosphatidylserine exposure on their membrane^[87]. PDMPs represent the most abundant MP population in humans, approximately 70%-90% of cell-derived MPs^[88]. Among them a large amount of PDMPs originate from megakaryocytes^[89]. PDMP production is enhanced *in vitro* by PLT agonists like Ca^{2+} , thrombin, ADP, collagen, fibrinogen, and high shear stress, confirming the statement that PDMPs are mainly derived by activated PLT^[86].

PDMPs are increased in autoimmune disorders such as mixed connective tissue disease, systemic sclerosis, primary Sjögren's syndrome, systematic lupus erythematosus, rheumatoid arthritis, Raynaud's phenomenon, and psoriasis^[87,90-92], as well as in cardiovascular diseases such as atherosclerosis, acute coronary syndrome, pulmonary embolism, and pulmonary arterial hypertension^[93-96]. Moreover, they can be used as antithrombotic indicators and side-effect markers following blood transfusion^[97,98].

Few studies have been conducted in IBD patients. Andoh *et al*^[99] showed increased PDMPs in active IBD patients compared to inactive ones and healthy controls. PDMPs correlated with clinical disease activity indices and PLT activity markers, and significantly reduced after remission achievement. However, this study included a small sample size for exporting safe conclusions and PDMPs were measured using ELISA and not flow cytometry, the latter being considered a more reliable method. Chamouard *et al*^[100] demonstrated that infliximab therapy induced a significant decrease in circulating MPs, mainly of PLT origin, in CD but not in UC, implicating that PDMPs shedding is important in the IBD inflammatory response. Finally, Palkovits *et al*^[101] noted that TF(+) MP and especially TF(+) PDMPs were significantly increased in IBD patients compared to healthy controls, although they didn't correlate with markers of coagulation activity and inflammation. These results indicate that PDMPs may have an important role in IBD. Taking into account the high procoagulant and proinflammatory predisposition of PDMPs, they can be useful targets, or even vectors, of future IBD therapies.

USE OF ANTI-PLATELET DRUGS IN IBD

Anti-PLT therapy is unanimously certified as evidence-based primary and secondary prevention therapy in high

risk cardiovascular patients resulting in reduced mortality rates^[102]. Based on existing evidence, one can assume that PLT could be an ambitious target cell for IBD therapies, as it represents the critical crossroad between inflammation and coagulation.

Clopidogrel is a potent suppressor of PLT activation, PLA formation and production of PLT activation markers such as P-selectin^[103-105]. Clopidogrel significantly inhibited PLT inflammatory markers and resolved IBD symptoms in rats after a single intra-colonic administration of trinitrobenzenesulfonic acid and oxazolone^[106]. Moreover, salicylic compounds like 5-aminosalicylic acid regimens, which are broadly used in IBD, significantly reduced PLT activation markers in IBD patients^[107]. However, the use of aspirin even in a low dose in IBD is still uncertain, as it is associated with exacerbation symptoms and should be offered in patients with a strong indication for it^[108]. Larger randomized controlled studies evaluating its systematic anti-inflammatory effect in IBD are needed in order to verify possible benefits.

Azathioprine and 6-mercaptopurine are reported to inhibit collagen, ADP, and arachidonic acid-dependent PLT aggregation, as well as PLA aggregate formation^[109]. GP II b/IIIa antagonists (eptifibatide, abciximab, and tirofiban) have been shown to be more competent in sCD40L down regulation compared to aspirin in high risk cardiovascular patients, an observation that might be proved useful in IBD^[110]. Moreover, infliximab therapy induced significant disruption of CD40/CD40L dependent cognate interactions^[111] and reduced circulated MPs^[100] in CD patients, suggesting a potent drug effect on TNF, CD40L, and MPs production in IBD.

Other studies evaluating anti-PLT activation marker products in experimental colitis have also been conducted. CD40/CD40L pathway inhibitor (Trapidil) administration resulted in a significant reduction of colonic inflammation in wild type murine DSS induced colitis^[74]. Moreover, CD40L deficient mice exhibited a reduced thrombotic response that was restored after sCD40L administration, highlighting the possible anticoagulant effect of anti-CD40L drugs in IBD where the risk for TE events is increased^[112]. Finally, P-selectin deficient mice or P-selectin, PSGL-1 blocking antibody utilization induced significantly decreased PLT recruitment in a DSS colitis mouse model^[113].

CONCLUSION

In conclusion, there are increasing data suggesting that PLT are important key regulators in inflammatory disorders beyond hemostasis and thrombosis. Inflammation, wound repair, angiogenesis, atherosclerosis, and tumor metastasis are only some examples that reveal PLT multifactorial role. In IBD pathogenesis, PLT activation could be the missing link between inflammation and coagulation, two "independent" processes linked in such a way that each one activates and propagates the other.

Thrombocytosis has been associated with IBD mani-

festations such as disease activity, iron deficiency anemia, and development of pouchitis, whereas PLT parameters (PDW, PCT, and MPV) have been suggested as surrogate markers for IBD. PLT count increase cannot be attributed only to inflammation, as we believe that iron deficiency should be considered a major governor of thrombopoiesis. Until now, no study was designed in such a way as to discriminate to what extent inflammation and iron deficiencies are responsible for PLT increase. However, particular interest should be given to iron replacement in IBD patients, and especially those with thrombocytosis and low inflammatory indices or/and low hematocrit. The possible association between iron replacement therapy and reduction of PLT activation markers raises new questions regarding the involvement of iron scarcity in the increased incidence of TE events in IBD patients, although the data are as yet inconclusive. Additionally, PLT parameters seem to display good predictive value regarding disease activity, and can be cautiously used as cost-effective follow-up biomarkers in IBD.

Despite the increasing number of studies revealing the dominant role of PLT in IBD, little has been clarified regarding the efficacy of anti-PLT drugs in IBD. Perhaps different existing pathways between PLT hemostasis and coagulation could explain the lack of potent anti-PLT drugs approved in IBD. Breaking this vicious cycle by encountering PLT inflammation properties appears to be a challenging ordeal for future investigators and clinical physicians, who will need to come up against resisting IBD flares with a reduced selection of effective drugs.

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WJG 20th Anniversary Special Issues (3): Inflammatory bowel disease

Update on nutritional status, body composition and growth in paediatric inflammatory bowel disease

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Abstract

Growth and nutritional status are important issues in paediatric inflammatory bowel disease (IBD). While linear growth is easy to assess, nutritional status is more complicated, with reports often compromised by the use of simple measures, such as weight and the body mass index, to assess nutritional status rather than more appropriate and sophisticated techniques to measure body composition. This review is an update on what is currently known about nutritional status as determined by body composition in paediatric IBD. Further, this review will focus on the impact of biologics on growth in paediatric IBD. Significant lean mass deficits have been reported in children with IBD compared with controls, and there is evidence these deficits persist over time. Furthermore, data imply that gender differences exist in body composition, both at diagnosis and in response to treatment. With respect to growth

improvements following treatment with biologics, there are conflicting data. While some studies report enhancement of growth, others do not. The relationship between disease severity, impaired growth and the requirement for biologics needs to be considered when interpreting these data. However, key features associated with improvements in growth appear to be successful clinical response to treatment, patients in early stages of puberty, and the presence of growth failure at the onset of treatment.

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Key words: Crohn's disease; Ulcerative colitis; Lean mass; Pubertal status; Infliximab; Inflammatory bowel disease

Core tip: Assessing body composition gives a much better indication of nutritional status than measures of anthropometry, such as BMI. In children with IBD, significant and persistent deficits in lean mass, suggestive of compromised nutritional status, have been reported, both at diagnosis and following treatment. Data pertaining to body composition in response to biologics is lacking, and data concerning growth improvements is controversial. However, evidence suggests that the key components associated with linear growth improvements when treating with biologics are (1) successful clinical response to treatment; (2) patients in early stages of puberty; and (3) the presence of growth failure at the onset of treatment.

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INTRODUCTION

Treatment considerations for paediatric patients with inflammatory bowel disease (IBD) are two-fold. Firstly, to achieve optimal disease control and attain remission, and secondly, to promote growth and progression through puberty. Often, when the first consideration is achieved, the latter will follow.

Growth failure and delayed puberty have long been associated with paediatric IBD, and is more prevalent in Crohn's disease (CD) than ulcerative colitis (UC)^[1]. Malnutrition has long been described as a factor contributing to growth impairment. The development of malnutrition in this cohort is multifactorial, being influenced by enteric nutrient losses, suboptimum intake, malabsorption of nutrients, and increased energy needs^[2]. However, while linear growth is straight forward to measure, and it is clear patients with CD are more prone to growth failure than UC, assessing malnutrition is more complex, with many results limited by crude measures of weight and body mass index (BMI) as proxy measures of malnutrition. Both these measures give little information about what is actually happening in the compartments of the body, and as such, increases in these parameters may not be representative of improved nutritional status.

Assessing body composition, that is, fat mass and lean mass at its most basic, gives a much better indication of nutritional status than anthropometry. With this in mind, growth and nutritional status in paediatric patients with IBD should be considered in terms of body composition, rather than simple anthropometric changes. This review is an update on what is currently known about nutritional status as determined by body composition in paediatric IBD. Further, as there have been several recent reviews about the prevalence and mechanisms of growth failure in IBD^[3,4] this review will focus on the impact of biologics on growth in paediatric IBD. Suitable research studies were identified from the literature by searching PubMed. Key words used to search included: IBD; body composition; nutritional status; growth; child; adolescent; infliximab. Relevant studies were also identified from the reference lists of search results.

NUTRITIONAL STATUS AS DETERMINED BY MEASURES OF BODY COMPOSITION

The techniques reported in the literature to measure body composition in IBD have paralleled the technical advancements in the field and become increasing more sophisticated and, therefore, accurate. Early work utilized anthropometry, such as measurements of skinfold thickness and body circumferences, which are proxy methods at best, and are less accurate than other methods like bio-electrical impedance (BIA), isotope dilution, total body potassium and dual energy X-ray absorptiometry (DXA).

IBD compared with normative data or controls

There is general consensus in the literature that lean mass

is reduced in children with IBD compared with controls. Boot *et al*^[5] reported reduced lean mass Z-scores in a cohort of 55 children with IBD, and this reduction persisted over two years of measurement. Similarly, Sylvester *et al*^[6] also found persistence of significantly lower lean mass Z-scores over two years in patients with CD, and these remained lower than controls even after adjustment for height. Werkstetter *et al*^[7] found significantly reduced lean mass in children with well controlled IBD compared with controls, as indicated by reduced phase angle α Z-scores measured by BIA. Our group^[8] has detailed significant reductions in body cell mass Z-scores (the metabolically active component of lean mass; adjusted for height) in patients with UC having repeated measures of total body potassium over three years. Lean mass adjusted for age and lean mass adjusted for height was shown by Burnham *et al*^[9] to be significantly lower in children with CD than controls, and in a regression model including height, age, Tanner stage and race, CD was associated with a 6% reduction in lean mass. Further, concurrent increases in fat and lean mass were reported in control subjects, whereas no relationship was found in those with CD; that is, increases in fat mass were not associated with increases in lean in children with CD. Azcue *et al*^[10] report body composition comparisons between patients with CD, healthy controls, and patients with Anorexia Nervosa, however, the different techniques used to calculate fat and lean mass, and the potential errors associated with this, limit the interpretation of these data. They do suggest that their finding of an elevated ECW:ICW ratio in CD compared with control subjects is indicative of protein-energy malnutrition, which in turn, is representative of lean mass deficits.

Despite consensus with respect to lean mass reductions in IBD, not all studies are in agreement with respect to fat mass. Boot *et al*^[5] suggest proportional reductions in lean and fat mass, as shown by percentage body fat that did not differ significantly from zero in their combined IBD cohort. In contrast, in an all CD cohort Burnham *et al*^[9] report fat mass adjusted for age and fat mass adjusted for height was not significantly different from controls. Similarly, in 42 children with CD weight gain over a two-year period was explained by gains in fat mass^[6].

Several studies highlight the ineffectiveness of BMI to determine nutritional status, as compared with body composition, in this cohort. Our group^[8] have recently shown normal BMI Z-scores in patients with UC, where body cell mass Z-scores were significantly reduced. Sylvester *et al*^[6] found children with CD had lean Z-scores consistently below the mean of healthy controls over two years, despite increases in BMI after 1 year that made them comparable to healthy children. In the study of Thayu *et al*^[11], changes in body composition were not reflected by changes in BMI, shown by normalisation of BMI in the face of continued significant deficits in lean mass at follow-up for female patients with CD compared with controls. While easy to calculate, BMI is of little value in determining nutritional status in children with IBD.

Gender differences in body composition

Several studies have detailed gender differences in body composition in patients with IBD, and further, have shown different treatment effects with respect to influence on nutritional status between genders. Dung *et al*^[12] report significantly higher percentage body fat in girls with CD compared with boys, and Sentongo *et al*^[13] detail girls with CD have significantly higher percentage body fat (approximately 6%) than controls, while boys were not different. However, for any given age, this study found significantly reduced lean mass in both the boys and girls with CD, which was associated with disease activity.

Work by Thayu *et al*^[11,14] is somewhat in disagreement with the body composition patterns described above. In contrast to the studies of Dung *et al*^[12] and Sentongo *et al*^[13], Thayu's group studied incident CD within two weeks of diagnosis, and report body composition at diagnosis^[14] and changes over time in response to treatment^[11] in the same cohort. At diagnosis, girls with CD displayed a decrease in both lean and fat mass compared with controls (wasting), while boys displayed reductions in lean mass, with relative preservation of fat mass (cachexia). After adjustment for race, Tanner stage, age and fat mass for height Z-scores, deficits in lean mass remained significant in both genders compared with controls, but were more pronounced in the girls with CD compared with boys, and within the girls, in the girls diagnosed during adolescence. Interestingly, body composition was not associated with disease activity, however, there were correlations with inflammatory markers. A subset of this cohort was followed for 24-63 mo, and in boys the body composition pattern changed from cachexia to one of normalised lean mass, but excess fat mass compared with controls. In girls, wasting at baseline developed into cachexia at long term follow-up, illustrated by continued deficits in lean mass, but normalisation of fat mass compared with controls.

An important consideration when interpreting these data detailing gender differences in body composition is the differential timing of peak height velocity between girls and boys^[15] and the changes in body composition that are associated. Gender differences may in part be explained by the timing of onset of disease in relation to the occurrence of peak height velocity^[13], which, in normal developing children, occurs earlier in girls than boys.

Effects of treatment on body composition

Data describing treatment effects of medications for IBD is somewhat confounded by disease severity. For example, disease activity was correlated with greater lean mass deficits in the study of Burnham *et al*^[9], and there was a trend for use of corticosteroids to be associated with lean mass reduction, which may simply be a result of increased corticosteroid use with more severe disease. The same study found mesalamine was predictive of lean mass for height Z-score less than -1.00, and the authors suggest this is indicative of upper gastrointestinal disease, which is associated with more micronutrient deficiencies,

and hence, may compromise nutritional status.

Nonetheless, Thayu *et al*^[11] described determinants of change in body composition during follow-up of their CD cohort. Medications included corticosteroids, methotrexate, 6-mercaptopurine, azathioprine, infliximab and enteral nutrition (not exclusive). In their model of predictors, greater improvements in lean mass for height Z-scores were associated with concurrent infliximab, while greater increases in fat for height Z-scores were associated with cumulative corticosteroid dose and methotrexate. Interestingly, high dose corticosteroid therapy has been shown to significantly increase whole body protein breakdown and loss, even in the short term, in children with CD and this may influence lean body mass acquisition in the long term^[16]. This potentially explains the persistent deficits in lean mass over time described by Thayu *et al*^[11] and Sylvester *et al*^[6].

Body composition in response to nutritional therapy

The early study of Lin *et al*^[17] combined measures of subscapular and triceps skinfold thickness, mid arm circumference, CT scanning of the thigh, and creatine excretion to investigate truncal and extremity body composition in children with both CD and UC combined. Normative data were not reported, nor were measures converted to Z-scores, but rather, their work investigated change in response to two durations of parenteral nutrition. Both short term (ST; 5 wk) and long term (LT; 10 wk) parenteral nutrition were associated with reduced disease activity and significant increases in weight, muscle mass, and truncal fat. Further, height was significantly increased at 50-d post cessation of ST nutrition and at cessation of LT, however, the relevance of this is limited as only absolute heights were given and not Z-scores (no control group). It is, therefore, unknown whether the increase in height was simply a reflection of normal growth, as opposed to increased growth, as there was no comparison group. With respect to extremity composition, increases in fat were more pronounced in the arms and increases in muscle were more pronounced in the legs, with changes more apparent with longer parenteral nutrition.

Investigating two types of exclusive enteral nutrition (EEN), Khoshoo *et al*^[18] also showed improved body composition and decreased disease activity. Fourteen children with CD increased weight, lean mass (BIA) and triceps skinfold thickness after both three and six weeks of EEN compared to baseline. Similarly, Azcue *et al*^[10] showed improvements in weight, percentage ideal body weight and absolute values of lean mass in children with CD on EEN. EEN was compared to corticosteroids and both groups significantly increased in the aforementioned parameters. In an age and Tanner stage matched subgroup of ten males, height was shown to significantly increase in the EEN group compared with the corticosteroid group. Interestingly, in both groups percentage of lean mass did not change significantly over the three months of treatment, but percentage fat mass did, with a trend to greater increase in the corticosteroid group.

This finding is perplexing, as for percentage fat mass to increase there would need to be a decrease in percentage lean mass. However, as previously mentioned several different body composition techniques, of varying accuracy and sophistication, were used to measure each component, and as there is error and assumptions inherent in different techniques, this questions the validity of their body composition component data comparisons. For example, Boot *et al*^[5] have shown that BIA overestimates fat mass compared with DXA as the standard in a cohort of IBD patients. They also found greater differences between the two methods when DXA determined bone mass and lean tissue mass were added together and compared with lean mass by BIA. Further, Sentongo *et al*^[13] have shown significant differences between lean and fat mass predicted from skinfold thickness compared with assessment by DXA.

GROWTH IN THE ERA OF BIOLOGICS

In the era of biologics, initial investigations into the efficacy and safety of their use in paediatrics are now evolving into interest in their ability to promote growth (Table 1) and improve nutritional status, although data predominantly investigate weight or BMI change and data on body composition are scarce. In three retrospective studies^[19-21], weight following infliximab therapy was shown to increase, but no significant changes in linear growth were reported. Afzal *et al*^[19] reviewed the case notes of 24 children and detailed growth parameters 6 mo prior to the first infusion of infliximab, at the time of first infusion, and 6 mo post third infusion. All children were on concomitant immunosuppression and while weight Z-score significantly improved from initial dose of infliximab to 6 mo post, no significant change in height Z-score was found between time points. Similarly, Sinitsky *et al*^[21] reported a significant improvement in BMI Z-score and a trend to improvement in weight Z-score at 1 year after starting infliximab in a cohort of 16 patients, however, height Z-scores were not different. Diamanti *et al*^[20] retrospectively evaluated 28 patients and divided them into groups according to therapy so as to compare combined infliximab, mesalazine and azathioprine, with mesalazine and azathioprine only. Significant increases in weight and BMI between baseline and follow-up (median 10 mo) were reported for the infliximab group, however, height was not found to be different in either treatment group.

Pfefferkorn *et al*^[22] described the relationship between growth and current treatment options in children remaining in Tanner stages 1-3 over 2 years. Thirty-six percent of their cohort received infliximab and no significant differences in height velocity Z-scores were found at one or two years follow-up. More frequent doses of infliximab were reported in children receiving early and sustained corticosteroid use, and this association persisted over the two-years of follow-up.

In contrast, other studies have reported resumption of normal linear growth following treatment with biologics^[23-29]. In a small number of patients with CD

($n = 6$) who were refractory to conventional therapy (corticosteroids and/or azathioprine) and had growth impairment (at least -1.00 change in Z-score for height), de Ridder *et al*^[25] described recommencement of normal linear growth velocity in half their retrospectively studied cohort. Borrelli *et al*^[23] prospectively studied 18 children with severe CD and reported both significantly increased weight and height Z-scores at 6 mo post induction regimen. Following the three induction infusions, endoscopic and histologic scores were significantly decreased. Clinical remission was achieved in 10 patients and inflammatory remission in 12 patients, and eight patients who had achieved both clinical and inflammatory remission had retreatment with infliximab beyond the induction regimen. When examining retreated patients compared with the 10 who only completed induction therapy, it was shown that the significant improvements in weight and height Z-scores remained only in the retreated group. It is interesting to note that mean height Z-scores were indicative of growth failure in the retreated group (-1.15), whereas they were not in the induction group (-0.86). Further, all patients in the retreated group displayed clinical and inflammatory remission post induction therapy. Hyams *et al*^[26] studied only patients who clinically responded to induction with infliximab in their randomised controlled trial of two different dosage regimens. Height Z-scores were determined only in those patients with greater than a 1 year delay in bone age, and at both wk 30 and 50 height Z-scores were significantly improved.

Pubertal progression and skeletal maturation are important considerations when evaluating the impact of therapies on growth. Both these parameters were taken into account by Walters *et al*^[29] in their retrospective investigation of growth during the first year of infliximab therapy. A bone age correction factor was applied to Z-score calculations for those children with a delay and patients were grouped according to pubertal status (Tanner stages 1-3 *vs* Tanner stages 4-5). All 27 patients with growth assessed established at least a partial response to the induction regimen, and mean height Z-score had decreased over the period from diagnosis to infliximab induction, even with the use of other conventional therapies. Height and height velocity Z-scores were subsequently found to improve only in those patients in early puberty, however, all children showed significant improvement in weight. Improvements in height velocity, weight and BMI were significantly greater in those children exhibiting complete symptomatic remission as opposed to partial. Similar results with respect to pubertal status and clinical response were reported in the retrospective study of Malik *et al*^[28]. Height velocity Z-scores accounted for pubertal status, and height and height velocity Z-scores significantly improved over the first 6-mo of treatment, with height Z-scores additionally showing significant increases 12-mo from baseline. Clinical responders showed significant improvements in height velocity. In a prospective study of children with severe refractory or corticosteroid dependent CD, ten children who had not completed pubertal growth showed significant improvement in height Z-score

Table 1 Summary of studies investigating the impact of biologics on linear growth

Ref.	Study type and biologic	Subjects and medication at baseline	Growth failure ¹	Pubertal status data	Measurement times	Remission achieved	Linear growth outcomes
Afzal <i>et al</i> ^[19]	Retrospective; infliximab	<i>n</i> = 24; median age: 10.3 yr; All concomitant immunosuppression	No	<i>n</i> = 0 in Tanner 5	T - 6; T0; T + 6 post 3 rd infusion	<i>n</i> = 17 clinical remission after 3 rd infusion; of these, <i>n</i> = 14 relapsed and required further infusions	No sig Δ ht Z at T + 6
Diamanti <i>et al</i> ^[20]	Retrospective; infliximab	<i>n</i> = 28; median age: 13 yr in infliximab, 5-ASA and azathioprine (Group A: <i>n</i> = 14); 14 yr in 5-ASA and azathioprine (Group B: <i>n</i> = 14)	Data not given	Data not given	T0; median 10 mo post	Clinical remission in group A	No sig Δ HV Z at 10 mo post for either group
Sinitsky <i>et al</i> ^[21]	Retrospective; infliximab	<i>n</i> = 16; mean age 13.0 yr; <i>n</i> = 2 concomitant MTX; <i>n</i> = 1 6-MP; <i>n</i> = 1 tacrolimus; <i>n</i> = 8 5-ASA; <i>n</i> = 14 azathioprine; <i>n</i> = 7 corticosteroid	No	Data not given	T0; T + 12	<i>n</i> = 10 clinical remission	No sig Δ ht Z at T + 12
Pfefferkorn <i>et al</i> ^[22]	Prospective; infliximab	Subgroup <i>n</i> = 34 commencing infliximab during first year of study; mean age: data not given; concomitant medication: data not given	No	Tanner 1-3	Dx; T + 12; T + 24	Data not given	No sig Δ HV Z at T + 12; No sig difference HV Z at T + 24 between infliximab ≥ 1 yr, vs < 1 yr or no infliximab
Borrelli <i>et al</i> ^[23]	Prospective; infliximab	<i>n</i> = 18; median age: 13 yr; <i>n</i> = 18 concomitant azathioprine; <i>n</i> = 15 mesalamine; <i>n</i> = 13 corticosteroids	Yes in retreated group only	No	T0; T + 6	After induction <i>n</i> = 10 clinical remission; <i>n</i> = 12 inflammatory remission. <i>n</i> = 8 were retreated	Sig ↑ ht Z from T0 to T + 6 in retreated group only; Note: all in retreated group had achieved clinical and inflammatory remission
Cezard <i>et al</i> ^[24]	Prospective; infliximab	Subgroup <i>n</i> = 10; mean age: data not given; concomitant medication: data not given	No	Pubertal growth not completed	T-12; T + 12	Data not given	Sig ↑ HV Z at T + 12
de Ridder <i>et al</i> ^[25]	Retrospective; infliximab	Subgroup <i>n</i> = 6 of refractory group; mean age: 13.8 yr; of these, <i>n</i> = 6 concomitant immunosuppression; <i>n</i> = 4 corticosteroids	Yes	No	Collection points unclear: patients followed for 8-122 mo	<i>n</i> = 3 good response; <i>n</i> = 2 became unresponsive at second infusion; <i>n</i> = 1 ceased due to allergy	<i>n</i> = 3 resumed normal linear growth velocity, all of which were in good response group; <i>n</i> = 3 no change
Hyams <i>et al</i> ^[26]	Prospective; infliximab, randomized to 8 or 12 weekly infusions	<i>n</i> = 103; mean age: 13.3 yr; however, ht Z only assessed in those with > 1 yr delay skeletal maturation (<i>n</i> = not reported); <i>n</i> = 93 concomitant 6-MP/azathioprine; <i>n</i> = 9 MTX; <i>n</i> = 56 5-ASA; <i>n</i> = 36 corticosteroids	Yes	> 1 yr delay skeletal maturation	T0; week 30; week 54	All displayed clinical remission to induction regimen prior to randomization	Sig ↑ ht Z from T0 to weeks 30 and 54
Malik <i>et al</i> ^[27]	Retrospective; adalimumab	<i>n</i> = 36; median age: 14.7 yr; of these, <i>n</i> = 34 prior infliximab (<i>n</i> = 7 non-responders; <i>n</i> = 16 loss of clinical response; <i>n</i> = 11 allergic reaction); <i>n</i> = 23 concomitant immunosuppression; <i>n</i> = 15 corticosteroids	No	<i>n</i> = 17 Tanner 1-3; <i>n</i> = 11 Tanner 4-5	T0; T + 6; <i>n</i> = 11 T + 12	<i>n</i> = 28 clinical remission	Sig ↑ ht Z and HV at T + 6 for whole group, those in clinical remission, Tanner 1-3, immunosuppression, allergic reaction to infliximab; no sig changes for group followed to T + 12; independent of corticosteroid use
Malik <i>et al</i> ^[28]	Retrospective; infliximab	<i>n</i> = 28; median age: 13.1 yr; <i>n</i> = 17 concomitant 5-ASA; <i>n</i> = 13 azathioprine; <i>n</i> = 13 MTX; <i>n</i> = 12 corticosteroids	Yes	<i>n</i> = 20 Tanner 1-3	T - 6; T0; T + 6; <i>n</i> = 25 T + 12	<i>n</i> = 21 clinical response; <i>n</i> = 10 clinical remission	Sig ↑ ht Z from T0 to T + 6, and T - 6 to T + 12 for whole group; Sig ↑ HV from T0 to T + 6 for whole group, clinical responders, Tanner 1-3, no corticosteroids, MTX throughout

Walters <i>et al</i> ^[29]	Retrospective; <i>n</i> = 27; median age: 14.3 yr; <i>n</i> = 3 infliximab concomitant corticosteroids; <i>n</i> = 25 immunosuppression	Yes	<i>n</i> = 9 delayed skeletal maturation; <i>n</i> = 19 Tan- ner 1-3; <i>n</i> = 8 Tanner 4-5	T0; T + 12; median 26 mo post (cur- rent)	<i>n</i> = 20 clinical remission; <i>n</i> = 7 partial remission	Sig ↑ HV from T0 to T + 12 for Tanner 1-3 (and this group displayed growth failure). Within Tanner 1-3, sig ↑ HV from T0 to T + 12 for complete remission; Sig ↑ ht Z from T0 to current for Tanner 1-3; ht Z negatively correlated with disease duration
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¹Growth failure defined as mean group height Z-score < -1.00 at pre or initial biologic infusion. ASA: Aminosalicic acid; MTX: Methotrexate; 6-MP: 6-mercaptopurine; Dx: Diagnosis; T - 12: 12 mo pre commencement; T - 6: 6-mo pre commencement; T0: Commencement of biologic; T + 6: 6 mo post commencement; T + 12: 12 mo post commencement; ht: Height; HV: Height velocity; sig: Significant at *P* < 0.05; Δ: Change; ↑: Increase; Z: Z-score.

in the year after treatment compared to the year before^[24]. In the whole group of 21 children, 90% achieve complete remission.

A further study by Malik *et al*^[27] detailed the effects of a different biologic on growth in children with CD, namely adalimumab. Their cohort comprised mainly of children (34 out of 36) who had previously been treated with infliximab but were either unresponsive, lost clinical response or had an allergic reaction. Both height Z-score and height velocity significantly improved over 6 mo, however, this increase was significant only in the group who achieved clinical remission. Further, height Z-score did not show significant change in those patients who were either unresponsive or lost clinical response to infliximab, but was only apparent in those with an allergic reaction to infliximab. Linear growth was also related to stage of puberty, with only those in the early stages of puberty (Tanner 1-3) showing significant increases in height Z-score and median height velocity, and while use of corticosteroids did not impact improvements in height, those on concurrent immunosuppression displayed significant improvement as opposed to those who were not.

In summary, growth deficits are a marker of more severe disease^[3], as is use of biologics^[30]. Hence, the relationship between treatment with infliximab and growth promotion seems multifactorial. From the data reviewed herein, features associated with improvements in growth with use of biologics appear to relate to clinical response to treatment, stage of puberty, and presence of growth failure. Evidence suggests that clinical response is important for improving growth and while limited data exist, this is probably related to mucosal healing^[23]. It is also apparent, and not surprising, that children late in puberty do not respond with linear growth improvement. This may have been a factor associated with the studies not showing improvement in height as pubertal status was either not assessed^[20,21], or indicated to be in the later stages^[19]. Better growth response is also seen in those patients who are suffering from growth failure prior to treatment, with studies showing no improvement involving a cohort where growth was not impaired^[19,21]. The study of Diamanti *et al*^[20] is limited by the authors only looking at change in actual height values, with both genders grouped together, and no information on pubertal status. Hence, it is difficult to determine at what stage

their patients are with respect to pubertal progression and peak height velocity.

CONCLUSION

Nutritional status, as indicated by compromised body composition (that is, reduced lean mass), is present in children with IBD and persists over time, irrespective of treatment. Further, alterations in body composition are expressed differently between boys and girls, and in response to treatment. Reports suggest girls present with wasting which morphs into cachexia with treatment. In contrast, boys present with cachexia, with resolution of lean mass with treatment, and excess of fat mass. It must be noted that literature in this area is relatively limited, and more studies are needed, particularly addressing responses to treatment.

As with compromised nutritional status, growth deficits are reported in children with IBD. Data are promising with respect to improvements in linear growth as a result of treatment with biologics, however, it is clear that further research is necessary in this area as the majority of studies conducted are retrospective in nature and subject numbers are small. Key features associated with improvements in growth appear to be successful clinical response to treatment, patients in early stages of puberty, thereby allowing a greater window of opportunity for growth potential, and the presence of growth failure at the onset of treatment, again allowing for greater growth potential. An area that is lacking for evidence is the impact of biologics on body composition, and more data are warranted in this area.

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WJG 20th Anniversary Special Issues (3): Inflammatory bowel disease

Inflammatory bowel disease course in Crohn's disease: Is the natural history changing?

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Abstract

Crohn's disease (CD) is a multifactorial potentially debilitating disease. It has a variable disease course, but the majority of patients eventually develop penetrating or stricturing complications leading to repeated surgeries and disability. Studies on the natural history of CD provide invaluable data on its course and clinical predictors, and may help to identify patient subsets based on clinical phenotype. Most data are available from referral centers, however these outcomes may be different from those in population-based cohorts. New data suggest the possibility of a change in the natural history in Crohn's disease, with an increasing percentage of patients diagnosed with inflammatory disease behavior. Hospitalization rates remain high, while surgery rates seem to have decreased in the last decade. In addition, mortality rates still exceed that of the general population. The impact of changes in treatment strategy, including increased, earlier use of immunosuppressives, biological therapy, and patient monitoring on the natural history of the disease are still conflictive. In this review article, the authors summarize the available evidence on the natural history,

current trends, and predictive factors for evaluating the disease course of CD.

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Key words: Crohn's disease; Natural history; Surgery; Mortality; Disease course; Inflammatory bowel disease

Core tip: Studies on the natural history of Crohn's disease (CD) provide invaluable data on its course and clinical predictors, and may help to identify patient subsets based on clinical phenotype. New data suggest the possibility of a change in the natural history in CD, with an increasing percentage of patients diagnosed with inflammatory disease behavior. Hospitalization rates remain high, while surgery rates seem to decrease in the last decade. Mortality rates still exceed that of the general population. The impact of changes in treatment strategy, including increased, earlier use of immunosuppressives, biological therapy, and patient monitoring on the natural history of the disease are still conflictive.

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INTRODUCTION

Crohn's disease (CD) is a chronic inflammatory disorder of the gastrointestinal tract of unknown etiology. Both genetic and environmental risk factors (*e.g.*, smoking or appendectomy) contribute to its pathogenesis^[1]. During

the past two decades, the incidence pattern of inflammatory bowel disease (IBD) has changed significantly^[2]. The disease course is reported to be highly variable, but the majority of patients eventually develop penetrating or stricturing complications. Nevertheless, there are still relatively limited data available on the natural history of IBD from population-based studies.

The phenotypic classification of CD based on clinical features plays an important role in patient management, and may help predict the clinical course in CD patients^[3]. In 2005, the Montreal revision of the Vienna classification system was introduced^[4]. The broad categories for CD classification remained the same [terminal ileum (L1), colon (L2) and ileocolon (L3) and upper gastrointestinal (GI) (L4) as modifier], behavior [non-stricturing non-penetrating (B1), structuring (B2) and penetrating (B3)] with some changes: *e.g.*, upper GI disease and perianal involvement became modifiers classified independently of, or alongside, disease at more distal locations and the later with disease behavior. Current practice guidelines from European Crohn's and Colitis Organisation advocate the use of the Montreal classification in both CD and ulcerative colitis (UC)^[5]. Using the Vienna classification system, it has been shown in referral IBD cohorts that a significant change in disease behavior often occurs over time, whereas disease location remains relatively stable^[6,7]. It is still uncertain whether this progression is preventable.

Other significant adverse outcomes include need for hospitalization, surgery, and reoperations. Hospitalization and surgery are considered to be markers of disease severity in CD and are associated with high costs^[8]. There are relatively limited data available on hospitalization trends, and data interpretation is complicated by local management strategy and reimbursement issues. According to recent population-based studies, major surgery was required in 40% to 50% of CD patients within 10 years of diagnosis in the last 2 to 3 decades, with postoperative recurrence rates as high as 50% at 10 years. However, new data suggest that surgical rates already began to decrease prior to the widespread use of biologicals. The ultimate negative outcome is mortality^[9]. CD mortality is still higher than that of the background population and current data do not suggest a change.

Recently, Peyrin-Biroulet *et al.*^[10] published a systematic review of the natural history of CD in population based-cohorts. According to the authors' conclusions, available data did not suggest a significant change in CD outcome, with approximately half of patients requiring surgery within 10 years of diagnosis. Furthermore, the authors stated that the impact of changing treatment paradigms with the increased use of immunosuppressants and biological agents on the natural history of the disease was poorly understood. In this article, evidence regarding the natural history, the current trends in outcomes and predictive factors for evaluating the disease course in CD, are discussed and summarized.

DISEASE LOCATION, BEHAVIOR AND OVERALL DISEASE ACTIVITY: CHANGING PATTERNS OR DIFFERENCES DUE TO DIAGNOSTIC TOOLS, AGE AT ONSET, GEOGRAPHIC REGION AND HOSPITAL SETTING?

In CD, disease location at diagnosis is relatively homogeneous and stable, with the exception of the reported variance in the frequency of upper gastrointestinal location, especially when comparing pediatric- and adult-onset populations. In addition, according to some studies, the proportion of isolated colonic disease seems to have increased in the last decade. In the recent study by the IBSEN group^[11], 27% of patients had L1 disease, 48% L2, 23% L3, and 2% L4 disease at presentation. Somewhat lower rates of isolated colonic disease were reported from Denmark (L2: 30%, 43%, and 37%, in 1962-1987, 1991-1993 and 2003-2004, respectively)^[12]. Similar data were recently reported from Eastern Europe (L1: 20%; L2: 35%, L3: 44%, and L4: 2.4%) in 2002-2006^[13]. Somewhat lower frequency of ileocolonic disease was reported from the Mayo Clinic^[14]. Disease extent was ileal in 45.1%, colonic in 32.0%, and ileocolonic in 18.6%.

Finally, two very recent, multinational, population-based cohorts have come to similar conclusions. In the EpiCom study^[15], the distribution was not significantly different in centers from Western and Eastern Europe (L1: 35% *vs* 43%, L2: 31% *vs* 24%, L3: 27% and 32%, isolated L4: 8% and 2%, total L4 involvement: 24% *vs* 17%). The frequency of total upper gastrointestinal involvement was higher compared to previous reports. Another study was published from eight countries across Asia and Australia^[16]. Interestingly, disease location was very similar in the Asian countries and Australia (L1: 31%, L2: 24%, L3: 45%, and L4: 5%). The highest variability is reported in the rate of upper gastrointestinal involvement. This may be at least partly associated with diagnostic procedures (*e.g.*, completeness of bowel investigation), but differences in the definitions and interpretation of minute upper gastrointestinal lesions. As an example, in the recent EpiCom study, only 10%-34% of adult onset patients underwent a gastroscopy, while a full colonoscopy was performed in 93%-96%. Additional small bowel imaging (capsule endoscopy, magnetic resonance imaging, or computed tomography) was performed approximately 60% of CD patients. Of note, location seems to be relatively stable with only 10%-15% change after approximately 10 years' follow-up^[7,17,18].

Up to one-third of patients present with complicated disease phenotype at diagnosis. In the IBSEN cohort, 36%, 49%, and 53% of patients presented with stricturing or penetrating disease at diagnosis or developed such complications within 5 or 10 years. However, some recent studies also reported a change in the initial disease behavior over time. In the Veszpremi cohort, patients

diagnosed from 1999 to 2008 presented more frequently with inflammatory disease behavior compared to the previous cohort (65% *vs* 50%)^[18]. Similarly, the probability of progression to complicated disease behavior was associated with the calendar year of diagnosis, but not with age at onset; after five and seven years 15.1% and 21.8% of patients diagnosed after 1998 progressed to complicated disease, while 27.4% and 33.3% of patients diagnosed between 1977 and 1998 showed such a progression. Other factors identified were disease location, perianal disease and smoking.

Recently, authors from New Zealand^[17] published a population-based cohort study, showing that > 70% of CD patients had inflammatory disease at diagnosis, while only 23% and 40% of patients with initial inflammatory disease progressed to complicated disease phenotypes after five and ten years of follow-up, respectively. The median follow-up for CD patients was, however, only 6.5 years. In a study from the Mayo Clinic, 81.4% had non-stricturing, non-penetrating disease, 4.6% had stricturing disease, and 14.0% had penetrating disease at diagnosis^[14]. Similarly, only 22% of patients had fistulizing complications in the Manitoba CD cohort^[19]. The cumulative risk of developing either complication in the Mayo cohort was 18.6% at 90 d, 22.0% at 1 year, 33.7% at 5 years, and 50.8% at 20 years after diagnosis. Similarly, B1 behavior was observed in 68% and 75% of patients in Western and Eastern Europe, respectively in the EpiCom study^[15] with 10% of all patients presenting with perianal involvement. The rate of inflammatory disease behavior was even higher in Australian patients in the ACCESS study^[16] (Australia: 88% *vs* Asian countries: 66%), with similar perianal involvement (12% and 18%). Another remarkable finding of this study was that UC incidence increased parallel with age. Nonetheless, some of these changes may result from bias due to diagnostic delay, differences in the diagnostic tools and completeness of bowel examination in the different time periods.

In contrast, in the landmark study by Cosnes *et al*^[6], up to 70% of CD patients developed either penetrating or stricturing disease within 10 years of diagnosis in a referral CD cohort. Similar results were published in a Belgian referral cohort^[17]. During 10 years' follow-up, 45.9% of patients had a change in disease behavior from non-stricturing, non-penetrating disease to either stricturing (27.1%) or penetrating (29.4%) phenotypes. In contrast, disease location remained relatively stable during follow-up, with only 15.9% of patients exhibiting a change in disease location within 10 years. The rate of perianal complication varies between 10%-20% at presentation. Of note, these were referral center cohorts and as highlighted earlier, trends were to some extent different in the population-based setting.

According to the available literature, pediatric-onset CD runs a more aggressive course, with more extensive disease location, more upper GI involvement, more active disease, growth failure, and need for more aggressive medical therapy in predominantly referral-center stud-

ies^[20-22]. While data on overall disease course so far have lacked consensus, pediatric disease behavior seems to parallel that of adults^[23]. A Scottish study simultaneously compared disease behavior and location in pediatric and adult onset IBD patients^[24]. In childhood-onset patients a clear difference in disease location at onset and after five years exists; with less ileum- and colon-only location but more ileocolonic and upper gastrointestinal involvement among pediatric-onset patients ($P < 0.001$ for each). In addition, disease behavior after five years did not differ between the two groups. Similar trends were recently reported from the Eurokids registry with a larger proportion of pediatric-onset patients presenting with extensive disease (L1: 16%, L2: 27%, L3: 53%, and L4: 54%)^[22]. Finally, according to French data, pediatric-onset CD was characterized by frequent occurrence of a severe phenotype during follow-up, with extensive location, complicated disease, and frequent need for immunosuppressives^[25].

Additionally, according to the findings by Pigneur *et al*^[21], patients with childhood-onset CD often have more severe disease, increased frequency of active periods, and increased need for immunosuppressants. In contrast, the cumulative risks of stricturing and penetrating complications and need for surgery were not different between childhood-onset and adult-onset patients. Similar findings were reported recently from a population-based study including both pediatric and adult onset cohorts from Hungary^[18] and another from Canada^[26]. Interestingly, in the most recent publication from the EPIMAD registry^[27], patients with pediatric-onset disease had roughly similar disease behavior at diagnosis compared with patients with an age at onset between 17-39 years, 40-59 year or > 60-years (B1: 72%, 66%, 69% and 78%). In this paper, pediatric-onset patients presented more frequently with ileocolonic disease, while elderly-onset CD patients (> 60 years at diagnosis), isolated colonic disease. In addition, complicated disease developed significantly more frequently in the pediatric-onset patients compared to patients with an elderly onset (50% *vs* 30% at maximal follow-up). The disease course in elderly-onset patients was altogether milder^[28]. Similar findings were reported also from Hungary^[29].

Few data are available regarding relapse rates and overall disease course in IBD. Most data were published from the Nordic countries. In one early publication, long-term disease course was reported in 185 CD patients followed-up regularly between 1960-1978 in Copenhagen^[30]. About 45% of patients were clinically asymptomatic for all observation years. The disease activity was low in approximately 30% of patients and moderate-to-high in approximately 25%. Continuous disease activity was observed in about 20% of patients and intermittent symptoms were reported in 35% of those with active disease in a given year. However, the cumulative relapse rate after five years reached 93.1%. Similar disease course was reported in a follow-up cohort from the same region in 1991-1993^[12].

Somewhat different rates were published in the EC-

IBD study^[31]. All-type first cumulative recurrence rates were 34%, 69.2%, and 77.5% after 1, 5, and 10 years of follow-up, respectively, in 358 CD patients, with similar second and third all-type relapse rates (40.2%, 76.9% and 82.6% *vs* 45.9 and 76.4% after 1, 5, and 10 years). Upper gastrointestinal location and therapy with 5-aminosalicylic acid therapy were associated with increased risk of relapse. Interestingly, relapse rates were associated with the geographic region. Higher relapse rates were reported from Copenhagen, while lower rates were observed in Greece, Italy, and Norway. Similar to earlier reports, high cumulative relapse rates (53%, 85% and 90% after 1, 5, and 10 years, respectively) were reported recently from the IBSEN group^[11]. This was associated with early need for steroids but not with disease phenotype or smoking habits. In contrast, approximately 44% of patients were in clinical remission during the second five-year period and 43% experienced a decrease in disease severity (according to predefined disease patterns) during the follow-up period. In contrast, 3% of patients experienced an increase in severity, 19% experienced chronic continuous symptoms, and 32% experienced a relapsing course.

HOSPITALIZATION: IS THIS AN OBJECTIVE MEASURE?

Although hospitalization is an important outcome measure, it is subject to inconsistency, as it is influenced by multiple factors other than disease severity, such as the need for diagnostic workup, health insurance reimbursement policies and ethnic differences. In addition, the threshold for hospitalization varies between specialized centers, community hospitals, and private practice. In addition, a restructuring of costs is currently seen, as highlighted in a short-term study from The Netherlands^[32]. In this study, tumor necrosis factor inhibitors (anti-TNFs) accounted for as much as two-thirds of the direct costs in CD and one-third in UC (with a three-month total cost of 1626€ in CD and 595€ in UC). Future studies are needed to investigate if tight control and aggressive therapy based on early patient profile stratification leads to superior long-term outcomes. A cost-benefit analysis is also required to justify the cost burden of these medications.

Relatively few data are available regarding hospitalization rates in patients with CD. Several decades ago, a significant proportion of diagnostics were performed on an inpatient basis, leading to fairly high initial hospitalization rates as reported in Scandinavia. For example, in Copenhagen the hospitalization rate in the year of diagnosis 83% in CD patients from 1962 to 1987. In addition, approximately 20% of patients were admitted yearly over the next five years^[33]. Data from the 1990s is available from a pan-European prospective follow-up study^[34]. This study confirmed that hospitalization rates declined significantly from the second year after diagnosis. The cumulative risk of overall hospitalization was also lower compared to the previous year (52.7% at 10 years from

diagnosis) but with considerable differences between countries. Rates were highest in Denmark, Ireland, Portugal while low rates were observed in Norway, Greece, and Italy.

Likewise, high hospitalization rates were reported in a population-based study from Canada^[35]. In 1994-2001, approximately 25% of subjects with Crohn's disease were admitted annually. The annual hospitalization rate declined from 29.2 to 26.9 per 100000 over the seven years of the study. The readmission rate was 39.4%, with almost half of the hospitalizations occurring for surgery. In a more recent population-based study from the same region^[36] the authors reported stable hospitalization rates in CD patients diagnosed between 1988 and 2008, with the highest hospitalization rates within the first year of diagnosis (approximately 1.3 admissions per person-year). Similar to previous studies, hospitalization rates declined after the first year by about half with a stable rate over the next 5 years.

A meta-analysis of hospitalization rates in IBD was published from nine European countries based on the data of the national statistic offices in 2009^[37]. Hospitalization rates varied significantly among countries, ranging between 1.2 and 4.3 discharges per 10000 for CD. The highest rates were found in Denmark (4.33) and Scotland (4.15), with the lowest in Spain (1.20), Switzerland (1.31) and the Netherlands (1.46), a trend partly unrelated to disease prevalence. Numbers were similar for UC and CD in the given country with a specific age-distribution pattern (CD: High peak in 20-30 year old patients and small peak in the elderly; UC: Opposite trend).

Finally, multiple studies investigating US national databases reported an increase in CD related hospitalization rates. However, it is difficult to determine if this rise is associated with disease prevalence, severity or both. According to the National Hospital Discharge Survey database, CD-related hospitalization rates increased significantly from 9.3 to 17.1 per 100000 from 1990 to 2003^[38]. In particular, hospitalization rates in the 45-64 year-old and > 65 year-old groups rose significantly, while rates in younger patients remained essentially unchanged^[39]. Similar trends were reported from the Nationwide Inpatient Sample^[40]. Hospitalization rates increased 4.3% annually between 1998 and 2004. In contrast, data from Kaiser-Permanente suggested a decrease in CD-related hospitalization rates by about one-third between 1998 and 2005^[41] parallel with an increased use of IBD related drugs (including a fivefold increase in anti-TNF use) and a shift in gastroenterology-related visits from the gastroenterology division to primary care.

In conclusion, although hospitalization patterns and causes may have changed, rates are still high, with approximately 50% of CD patients requiring hospitalization within 10 years of diagnosis. Actual rates may vary significantly among age groups, time periods, reimbursement settings, and among countries. Findings must be interpreted with attention given to the context of disease prevalence, treatment strategy, and health care access.

SURGERY IN CROHN'S DISEASE: RATES, TRENDS AND CAUSES

Surgery is one of the most objective outcome measures, since it is only performed if clinically indicated. Almost decade ago, partly based upon historical data, the probability of surgery was reported between 3% and 96% within 15 years of diagnosis^[42], with clinical relapse and reoperation rates of 50%-60% and 28%-45%, respectively, during the subsequent 15 years. Surgical resection rates over time vary widely among published studies, ranging between 25% and 61% in the first five years. Early studies reported extraordinarily high surgical rates, as high as 30%, 50%, and 60% at 5, 10, and 15 years, respectively, in the population-based Stockholm County cohort from 1955-1974^[43]. Surgical rates did not seem to change according to an update from the same cohort^[44]. Even higher rates were reported some years later in a population-based cohort from Denmark^[45], with up to 35% of CD patients requiring surgery in first year after diagnosis. The cumulative surgery rate was 61% and 82% after 10 and 20 years.

Lower surgery rates were reported in the pre-biologic IBSEN cohort^[11]. In patients diagnosed between 1990-1994, surgery rates of 14%, 27%, and 38% at 1, 5, and 10 years were observed. Similar surgery rates were reported from the multinational European EC-IBD cohort diagnosed in the same time period with a cumulative surgery rate of 37.2% after 10 years and reoperation rates of 2.2%, 18.5%, and 35.9% at 1, 5, and 10 years, respectively^[51]. A geographic variability was reported. Patients from northern European centers, especially Copenhagen, had higher surgical need due partly to differences in disease phenotype. Interestingly, cumulative surgery rates were comparable from a recent publication from a referral center in South Korea^[46], which reported data from 1991 to 2007, which showed cumulative probability of surgery of 15.5%, 25.0%, and 32.8%, at 1, 5, and 10 years after diagnosis, respectively. Surgery rates in referral center may not be directly comparable with that reported from population-based studies, however. Geographic variability is also evident in Asia, as surgery rates were much higher in a Japanese referral center cohort^[47], reaching as high as 37.6%, 60.4%, and 74.2% at 5, 10, and 15 years. This is comparable to historical studies from Europe in the 1960s and may represent a distinct patient management strategy.

An association with disease phenotype was reported in multiple studies. Terminal ileal location, stricturing or penetrating disease, and younger age at diagnosis (< 40 years) were identified as risk factors for surgery. Recent data from Canada, Denmark, the United Kingdom, and Hungary, however, suggest that surgical rates were falling (Table 1) prior to the advent of biologic therapy, as summarized by the IOIBD Epidemiology Task Force report^[8]. This trend is best highlighted by a Danish study^[12]. The rate of early surgery (within one year of diagnosis) fell from 35% to 12% between 1962 and 2004. Risk has

Table 1 Surgery trends for Crohn's disease in population based cohorts by years from initial diagnosis

Geographic region and time period of investigation	Time from diagnosis		
	1 yr	5 yr	10 yr
North America/Asia			
Olmsted County, MN, United States ^[38]			
1970-2009		38%	48%
Manitoba, Canada ^[2]			
1988-2008	13%	24%	32%
2001-2008	10%	18%	
South Korea ^[39]			
1991-2007	15%	25%	33%
Europe			
Sweden ^[21]			
1955-1974		30%	50%
Denmark ^[25-37]			
1960-1978	35%		61%
2003-2005	12%		
Denmark ^[51]			
1979-1986		44.70%	
2003-2011		19.60%	
Norway ^[28]			
1990-1994	14%	27%	38%
Wales, United Kingdom ^[32]			
1986-1991	32%	59%	
1992-1997	25%	37%	
1998-2003	19%	25%	
Veszprem Province, Hungary ^[32,33]			
1977-2008	15%	31%	52%
2002-2006	10%	21%	
EC-IBD ^[29]			
1991-2003			40%

¹Referral cohort.

continued to decline, parallel with increased use of immunosuppressives and biologicals, although causality was not established^[48]. Similar trends were reported in a population-based CD cohort from Manitoba, Canada^[37,38]. Surgery rates at one and five years decreased from 13% and 22% in patients diagnosed between 1996 and 2000 to 10% and 18% for those diagnosed between 2001 and 2008 (HR = 0.79; 95%CI: 0.65-0.97). Reoperation rates were unaffected by the era of diagnosis. In contrast, high operation rates were reported from the Mayo Clinic^[10] in patients diagnosed between 1940 and 2001 with a cumulative risk for surgery 24%, 49%, and 64% at 1, 10 and 30 years from diagnosis, respectively. In an update of the same cohort, presented in an abstract form, surgery rates did not seem to decline in patients diagnosed between 1970 and 2004 with 38%, 48%, and 61% of patients being operated on at 5, 10, and 30 years.

An association was also suggested with a change in disease management including tight follow-up and early immunomodulator therapy, however data are partly conflicting. In a previous referral center study from France, the need for intestinal surgery did not decrease despite increased use of immunosuppressants^[49]. However, in this study immunosuppressives were almost exclusively started after surgery. In contrast, recent population-based reports from Wales and Hungary^[50,51] reported that early azathioprine (AZA) use may be associated with reduced

frequency of resective surgery. In the study from Wales, surgery rates decreased from 59% to 25% at five years after diagnosis between 1986 and 2003. A similar five-year surgery rate (21.3%) was reported in the latter study in patients diagnosed between 2002 and 2006^[13]. In addition, a French study reported an association between the duration of anti-TNF and AZA therapy and risk for surgery^[52]. Of course, long treatment duration allows responders to the above therapies to be identified.

While data are mixed and there exists geographic variation, recent data suggest a multifactorial trend for decreasing surgery. Disease behavior at diagnosis as reported in the most recent studies is more often inflammatory compared to earlier CD cohorts^[15,16,18]. In addition, diagnostic tools and follow-up strategy has changed significantly in the last decade, parallel with the earlier and more widespread use of immunosuppressives, as reported in a recent publication from Canada^[37]. In this study, authors reported an association between early gastroenterologist care and lower risk of surgery parallel with an increased early use of immunosuppressives. However, exposure to immunosuppressives is still relatively limited in the population-based studies and reoperation rates are essentially unchanged.

However, results from two recent prospective randomized clinical trials cast some doubt on the efficacy of early thiopurine therapy. In the first paper, the GETAID group^[53] reported that early aggressive therapy with AZA (2.5 mg/kg) within 6 mo of diagnosis was no more effective than conventional management in increasing time of clinical remission as assessed by trimesters for 36 mo. However, 61% of the patients in the “conventional” group required AZA within a median of 11 mo of diagnosis, which cannot be interpreted as a conservative approach. Therefore, a more accurate interpretation is that authors compared early aggressive strategy with an early-accelerated strategy, and still the need for perianal surgery was lower (4% *vs* 18%, $P = 0.036$). Another study, AZ-TEC, from the Spanish IBD group^[54], appears promising in design; early CD patients (< 8 wk of diagnosis), after entering remission, patients were randomized to receive AZA or placebo. The endpoint was steroid-free remission at week 76. Unfortunately, the trial was stopped for futility; therefore the power of the study is somewhat questionable.

A more precise interpretation of the results reveals difficulties. First, diagnosis can still change in approximately 10% to 15% of CD patients during follow-up, as suggested the IBSEN group. Thus, 8 wk from the first specialists visit and diagnosis may introduce some unintentional bias with regards to the above. Second, we must assume that 30% of patients entered remission without steroid therapy, since under standard steroid taper schedules patients treated with steroids at diagnosis should still have received steroids at 8 wk. In addition, approximately one fourth of patients entered the trial without clinical remission. In contrast, median C-reactive protein (CRP) was low (CRP at diagnosis was not given). Of note, 92%

of patients had inflammatory disease, extensive location was observed in only one-third of patients, and patients with fistulizing (internal penetrating or perianal fistula) or stenosis were excluded. Thus we propose an alternative interpretation of the findings: mild phenotype patients at diagnosis do not necessarily benefit from early AZA therapy in the short term. However, this trial does not provide data on the efficacy of early AZA therapy in patients with complicated disease phenotype at diagnosis, nor whether AZA has the potential to change the natural history of the disease. In addition, the definition of clinical relapse was based simply on CD activity index (CDAI) and this does not adequately define steroid-free status, since under this definition most patients would have a relapse as defined by a CDAI elevation before they would need steroids. This is indeed a very soft endpoint. Interestingly, with a modified definition of relapse (CDAI > 220) AZA patients had a significant clinical benefit, even bearing in mind the limitations of CDAI. From this trial, it should be clear that the use of CDAI is insufficient as the only definition of relapse. Other objective parameters are needed, such as a change in CDAI > 100 from baseline, a need for a change in the medical therapy, or the development of complications. Development of complicated disease or need for surgery would be the optimal outcome measures to study the natural history of the disease.

Of note, surgery should not always be regarded as a negative outcome, and it has an important place in the management of CD patients. Early surgery has been shown to prolong clinical remission (HR = 0.57; 95%CI: 0.35-0.92)^[55]. In addition, CD patients with limited complicated terminal ileitis diagnosed at surgery were reported to have low reoperation rates, and needed less steroids and immunosuppressants during follow-up than those not diagnosed intra-operatively^[56]. The same was proven for early terminal ileum resection in a population-based Hungarian cohort^[57]. In these patients, surgery is part of a proactive treatment strategy and possibly represents an alternative to medical therapy. On the other hand, surgery during the first 6-10 mo of diagnosis is clearly linked to unavoidable complications already present at diagnosis. Unfortunately, this is more representative of the initial cohort characteristics and should not be interpreted as a real outcome measure. Thus, if we would like to study the association between management and treatment strategy most probably these patients should be excluded from the analysis. Finally, the above surgery rates and trends were reported from the pre-biologic era in cohorts with no or only minimal or anti-TNF/biological exposure. Whether biological therapy directly influences long-term surgery trends outside of clinical trials remains unclear.

MORTALITY

In a meta-analysis from 2010, mortality in CD was increased with a pooled standardised mortality (SMR) of 1.39 (95%CI: 1.30-1.49)^[58]. The meta-analysis included

Table 2 Key issues on the natural history of Crohn's disease

-The distribution of location in Crohn's disease (CD) has not changed significantly in the recent decade, but differs according to age at onset
-Recent data indicate that there are an increasing proportion of Crohn's disease patients are diagnosed with an inflammatory disease behavior. The progression to complicated disease phenotype is decreased
-There is evidence from population-based studies that the surgery rates have recently declined in Crohn's disease
-Data suggest that the decline in the surgical rates is partly associated with early use of thiopurines. However, the relative importance of changes in treatment strategy and patient monitoring on the natural history remain conflictive
-Overall mortality rates in CD have been higher than that in the background population, and there is only little evidence that these have changed in the last decade. In addition, an increased mortality from gastrointestinal causes is constantly reported
-Further data are needed to assess whether tight, and objective patient monitoring (including clinical, laboratory, endoscopy and imaging) or early administration of biological would lead to superior outcomes
-Cost-effectivity of the new treatment and monitoring strategies has to be established

nine population-based studies of which eight were European (including an EC-IBD study). Causes identified were cancer, COPD, gastrointestinal disease, and genitourinary disease. A recent nationwide study from Denmark confirmed a 50% increased mortality in CD, and concluded that mortality in CD did not decrease over time, despite a change in patient management^[59]. Similar results were published some years earlier in another meta-analysis^[60], which included referral center data. In subgroup analyses, the SMR ratio was increased in hospitals (SMR = 1.73; 95%CI: 1.45-2.47), referral centers (SMR = 2.06; 95%CI: 1.63-2.60), and population-based studies (SMR = 1.48; 95%CI: 1.28-1.70).

In contrast, the authors of two very recent population-based studies failed to confirm an increase in the overall CD mortality. In a study from Finland, mortality was not increased in 1915 adult IBD patients in 1986-2007. Mortality was increased from diseases of the digestive system, but there was a reduced mortality from mental and alcohol-related behavioral disorders compared to the general Finnish population^[61]. Another recent population-based study from South-Limburg, in the Netherlands did not find increased overall mortality in CD between 1991 and 2003 (SMR = 1.1; 95%CI: 0.7-1.6), despite increased mortality from gastrointestinal causes (SMR = 7.5; 95%CI: 2.8-16.4) in this patient group^[62]. This concurs with previous reports from the Mayo Clinic, where authors did not find increased mortality in 314 patients between 1940-2001 (SMR = 1.2; 95%CI: 0.9-1.6)^[63]. In addition, an increased risk of dying from non-malignant gastrointestinal causes (SMR = 6.4; 95%CI: 3.2-11.5), gastrointestinal malignancies (SMR = 4.7; 95%CI: 1.7-10.2), and COPD (SMR = 3.5; 95%CI: 1.3-7.5) was also observed. In contrast, another study from Kaiser Permanente reported increased mortality in CD patients between 1996 and 2003 (SMR = 1.4; 95%CI: 1.2-1.6)^[64]. In conclusion, there is insufficient evidence to support the hypothesis that overall CD mortality trends

has changed, it is slightly increased together with a consistently increased mortality having been reported from gastrointestinal causes.

SUMMARY AND CONCLUSION: IS THE NATURAL HISTORY OF CD CHANGING?

Studies on the natural history of CD provide invaluable data on the disease course as well as clinical predictors, and may help identify patient subsets based on clinical phenotype. Most data are available from referral centers, however outcomes are different from data reported from population-based cohorts, so that results are not directly comparable.

New data suggest a possible change in the natural history of Crohn's disease (Table 2), with increasing numbers of patients diagnosed with inflammatory disease behavior, likely, one would hope, due to new diagnostic techniques and tools. Hospitalization rates remain high, yet hospitalization is a relatively soft endpoint, and actual rates may vary significantly according to age group, reimbursement setting, and countries. Findings must be interpreted with attention to disease prevalence, treatment strategy, and health care access. In contrast, surgery rates seem to have decreased in the last decade, yet it is difficult to identify the drivers of this change. A combination of the greater proportion of patients with uncomplicated disease behavior, changes in patient monitoring, different therapeutic strategies, and altered attitude towards surgery may be at least partly responsible. Finally, mortality rate in CD still exceeds that in the general population and there is only little evidence that this has changed.

The impact of changing treatment strategy on the above trends, including increased, earlier use of immunosuppressives and biologicals, and changed systems for patient monitoring on the natural history is not entirely clear. Unfortunately, data from randomized clinical trials are of limited value in studying the natural history of the disease. This is partly because follow-up is limited in duration and open-label extensions include the same confounders as population-based cohorts. In addition, the patient populations do not reflect the patients from everyday clinical practice, as highlighted by a recent paper from the United States^[65]. Therefore, a direct extrapolation of the findings to the clinic is often difficult.

In conclusion, for clinical practice, it is important to use available results from the published literature. We must identify markers of progressive as well as mild disease, since an early patient stratification enables clinicians to select the most appropriate therapy for a given patient. Further data are needed to investigate whether tight, objective patient monitoring and early administration of biological agents lead to superior outcomes. Some clinical trials are underway (CALM, REACT) and results will be available soon. However, the cost-effectiveness of the new treatment and monitoring strategies also must be established in the near future. In addition, it will be extremely important to follow-up the recent multinational,

multicenter, population-based patient cohorts (EpiCom and ACCESS), since accurate long-term data on harder endpoints such as complications, surgery, and ultimately mortality in the biological era is urgently awaited, and can be obtained only in this setting. The key factor is the appropriate adjustment for confounders.

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WJG 20th Anniversary Special Issues (3): Inflammatory bowel disease

Interleukin and interleukin receptor gene polymorphisms in inflammatory bowel diseases susceptibility

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Abstract

Inflammatory bowel disease (IBD), which includes Crohn's disease (CD) and ulcerative colitis (UC), represents a group of chronic inflammatory disorders caused by dysregulated immune responses in genetically predisposed individuals. Genetic markers are associated with disease phenotype and long-term evolution, but their value in everyday clinical practice is limited at the moment. IBD has a clear immunological background and interleukins play key role in the process. Almost 130 original papers were revised including meta-analysis. It is clear these data are very important for understanding the base of the disease, especially in terms of clinical utility and validity, but text often do not available for the doctors use these in the clinical practice nowadays. We conducted a systematic review of the

current literature on interleukin and interleukin receptor gene polymorphisms associated with IBD, performing an electronic search of PubMed Database from publications of the last 10 years, and used the following medical subject heading terms and/or text words: IBD, CD, UC, interleukins and polymorphisms.

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Key words: Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Interleukin; Interleukin receptor; Polymorphisms

Core tip: Inflammatory bowel diseases (Crohn's disease and ulcerative colitis) are chronic, progressive disorders of the gastrointestinal tract. Different genes, including interleukin genes play central role in mediating and modulating of inflammation in inflammatory bowel diseases. In this review we summarized the interleukin and the interleukin receptor genes associated with Crohn's disease and/or ulcerative colitis performing an electronic search on the PubMed database focusing on the following terminology: inflammatory bowel disease, Crohn's disease, ulcerative colitis, interleukin and interleukin receptor.

Magyari L, Kovcsdi E, Sarlos P, Javorhazy A, Sumegi K, Melegh B. Interleukin and interleukin receptor gene polymorphisms in inflammatory bowel diseases susceptibility. *World J Gastroenterol* 2014; 20(12): 3208-3222 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i12/3208.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i12.3208>

INTRODUCTION

Inflammatory bowel disease (IBD) - clinically classified as Crohn's disease (CD; OMIM 26600) and ulcerative

colitis (UC; OMIM 191390) - is a common chronic, relapsing inflammatory disorder of the gastrointestinal tract^[1]. In Europe the highest annual incidence of CD is 12.7/100000 and 24.3/100000 for UC. In Asia and in the Middle East both rates are much lower (CD: 5.0/100000 and UC: 6.3/100000). However in North America the incidence for UC is 19.2/100000 and they have the highest rate for CD in the world with 20.2/100000^[2]. Although the precise etiology of IBD still remains obscure, the accepted hypothesis is that in genetically predisposed individuals the commensal luminal flora trigger an inappropriate, overactive mucosal immune response causing intestinal tissue damage that is further modified by specific environmental factors (*e.g.*, smoking)^[3].

The location of inflammatory lesions and the types of cytokines involved in the pathogenesis mainly distinguish CD from UC. Whereas CD is a segmental, transmural disorder involving any part of the gastrointestinal tract, UC is characterized by superficial, continuous mucosal ulcers restricted to the colon. Imbalances between pro- and anti-inflammatory cytokines in the mucosa have been established for both CD and UC^[4]. CD is associated with a T helper type 1 (Th1)^[4] and T helper type 17 (Th17)^[5] immune response, thus interferon gamma/interleukin 12 (IFN γ /IL12) and IL23/IL17 cytokines assign the downstream release of complex network of further pro-inflammatory cytokines (*e.g.*, IL18, IL2, IL1, IL21, IL22) (Figure 1). Th17 and a modified Th2 cytokine profile (IL13 and IL5) are characteristic for UC. In addition, IL6 and tumor necrosis alpha (TNF α) are produced by both Th1 and Th2 cells as well as by macrophages in both IBD entities. A further group of T cells, regulatory T cells (Treg) cells are important for the control of immune responses to self-antigens preventing autoimmunity and maintaining self-tolerance^[6]. The final result of this activated cytokine network is the recruitment of more effector cells and the beginning of mucosal inflammation, which will eventually become chronic due to defective regulation of the immune response^[6].

First, genome-wide association studies (GWAS) resulted in the identification of many novel susceptibility loci CD and later for UC^[7,8]. To date, the number of known risk loci has expanded to 163^[9]. Some loci seem to be specific either to CD or to UC, whereas others confer common susceptibility to IBD; approximately 30% of IBD-related genetic loci are shared^[10,11]. The IBD-associated loci encode genes involved in innate pattern recognition [nucleotide-binding oligomerization domain-containing protein 2 (NOD2), autophagy (autophagy-related protein 16-1 (*ATG16L1*), immunity-related GTPase family M protein (*IRGM*), differentiation of Th17-T lymphocytes (*IL23R*), maintenance of epithelial barrier integrity IBD5 locus], and coordination of adaptive immune responses [human leukocyte antigen (HLA)-region]^[12]. Polymorphisms in genes encoding cytokines and cytokine receptors may affect the course of the inflammatory cascade and thereby increase the risk of developing IBD.

In this review we discuss in each of the IL families

only those interleukins or interleukin receptors in detail, which have relevant polymorphisms in IBD, CD or UC (Figure 2).

SYSTEMATIC REVIEW

We conducted a systematic review of the literature of the last 10 years on interleukin susceptibility genes to IBD. PubMed was searched for papers and abstracts published in English-language journals. We used the following medical subject heading terms and/or text words: “inflammatory bowel disease”, “ulcerative colitis”, “Crohn’s disease” and “cytokines”. The search was focused on interleukin susceptibility genes polymorphism resulting in IBD. No restrictions were placed on race, ethnicity, or geographic area. Extraction from each study was conducted independently by all authors, and consensus was achieved for all data.

INTERLEUKIN AND INTERLEUKIN RECEPTOR GENE POLYMORPHISMS

ILs are the subset of a larger group of cellular messenger molecules called cytokines, which are humoral, small (4-15 kDa) inducible immune-regulatory proteins or glycoproteins which mediate communication between cells, regulate cell growth and differentiation, and play a central role in the development and homeostasis of the immune system^[6]. They act on target cells by binding to specific IL receptors, initiating signal transduction and second messenger pathways within the target cell. This can result in gene activation, lead to mitotic division, growth and differentiation, migration, or apoptosis. Cytokines act in a highly complex coordinated network in which they induce or repress their own synthesis as well as that of other cytokines and cytokine receptors. The nomenclature of ILs is continuously evolving (www.genenames.org/genefamilies/IL); they are assigned to each family based on sequence homology and receptor chain similarities or functional properties (Table 1)^[13].

IL1 FAMILY

The IL1 family is a group of 11 cytokines (IL1A, IL1B, IL1RN, IL18, IL33, IL36A, IL36B, IL36G, IL36RN, IL37 and IL38), which have similar gene structure and induce a complex network of proinflammatory cytokines. The interleukin 1 receptor (IL1R) family also expands to 9 distinct genes and includes coreceptors, decoy receptors, binding proteins, and inhibitory receptors^[14].

IL1

IL1 is a potent proinflammatory cytokine, which affects cell proliferation, differentiation, and the function of many innate and specific immunocompetent cells, and acts as an endogenous pyrogen. It also mediates many inflammatory diseases by initiating and potentiating im-

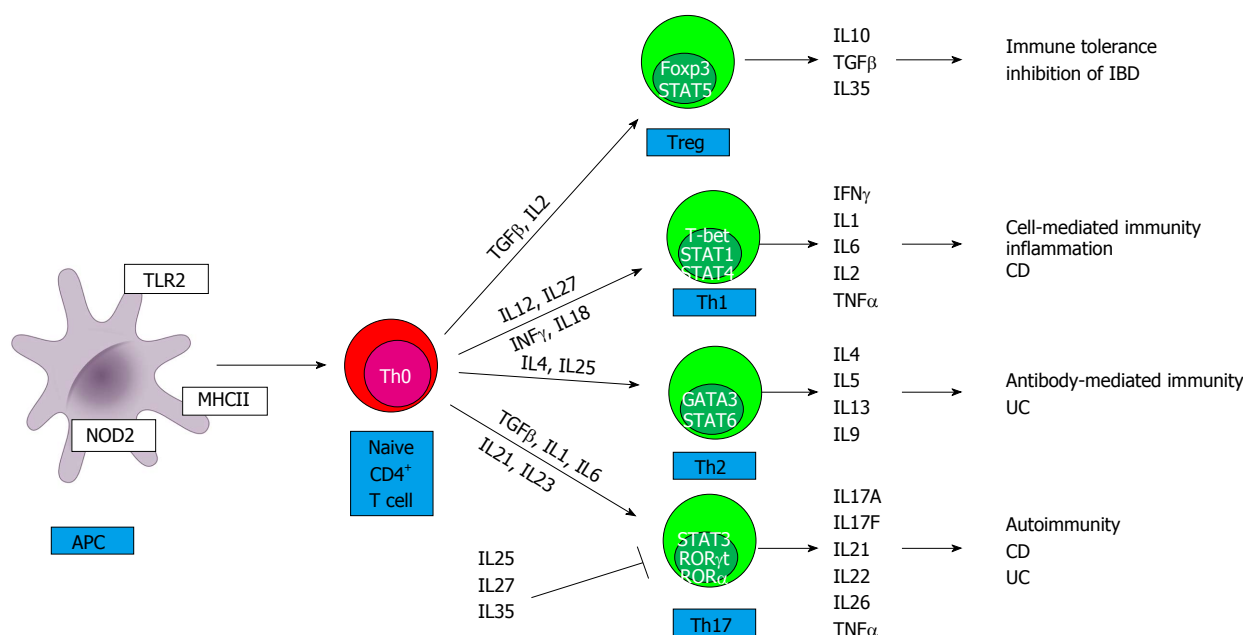


Figure 1 Differentiation of effector T helper and regulatory T cells in inflammatory bowel disease. Antigen presenting cells (APCs) (*i.e.*, macrophages and dendritic cells) in the lamina propria are increased in absolute number in both forms of inflammatory bowel disease (IBD). First, microbial products (pathogen associated molecular patterns, PAMPs) bind to a group of detection molecules of the innate immune system, called pattern recognition receptors (PRRs). This includes Toll like receptors (TLRs) on cell surface intracellular compartments, and the cytoplasmatic nucleotide-binding oligomerization domain-containing protein (NOD)-like receptors (NLR) family. Stimulation of these receptors induces intracellular signaling cascades, resulting in secretion of large number of cytokines, chemokines, and immunomodulatory factors. APCs interact with T cells by presenting an antigen on the surface of the major histocompatibility complex II (MHCII), which is recognized by the appropriate T cell receptor. The development of T helper (Th)1, Th2, Th17 and regulatory T cells (Treg) subsets from naive, Th0 cells during primary immune response is mainly determined by this cytokines and chemokines. It is under the control of certain transcription factors: T-box expressed in T cells (T-bet), GATA binding protein (GATA3), retinoid-related orphan receptor (RORγt), RORα, signal transducer and activator of transcription (STATs) and forkhead box P3 (FoxP3). Interleukin (IL)12 is the hallmark cytokine for Th1 cell lines, which produce interferon gamma (IFNγ) and are important for host defense to intracellular pathogens. IL4 promotes differentiation into Th2 cells, which produce IL4, IL5, and IL13 and participate in controlling humoral immunity to extracellular parasites and allergic inflammatory responses. Th17 cells develop from naive T cells in the presence of transforming growth factor beta (TGFβ), IL23, IL1B or IL6. The effector cytokines IL17A, IL17F, and IL22 play key roles in Crohn's disease (CD) and ulcerative colitis (UC), and in other autoimmune diseases. TGFβ and IL2 together convert naive T cells into regulatory T cells, which promote self tolerance and prevent autoimmunity. CD is a predominantly Th1 and Th17-mediated disorder, while UC is associated with a Th17 and a modified Th2 cytokine profile.

immune and inflammatory responses^[13].

IL1 is made up of two major proteins: IL1A (OMIM 147760) and IL1B (OMIM 147720)^[15]. These proteins exert similar effects, first, by binding to the first extracellular chain of the IL1 type I receptor (IL1RI) (OMIM 147810) that recruits the IL1 receptor accessory protein (IL1RAP) (OMIM 602626), which serves as a coreceptor and is necessary for signal transduction. IL1A and IL1B are also able to bind to the IL1 type II receptor (OMIM 147811), which acts as a decoy receptor and is not involved in signal transduction^[13].

IL1B has an important role in initiating and amplifying the inflammatory response^[16]. Normal colonic mucosa cells produce very little mature IL1B, however in the mucosa of affected IBD patients, a large amount of mature IL1B is produced^[17,18]. The inability of normal intestinal macrophages to produce mature IL1B could result from regulation at one or more steps from gene activation to post-translational processing of the propeptide by IL1B converting enzyme and release of the mature peptide^[19,20].

The IL1 receptor antagonist (IL1RN) (OMIM 147679) is an anti-inflammatory cytokine, which is synthesized and released in response to the same stimuli that lead to

IL1 production^[21]. IL1RN lacks the IL1RAP interacting domain, so that binding of the IL1RN to IL1RI inhibits IL1 signaling^[15]. In IBD and several other inflammatory diseases, an imbalance the IL1RN/IL1 ratio contributes to the chronic inflammatory response^[22-24]. Polymorphisms of the *IL1RN* gene, which can lead to changes in the IL1RN and IL1 balance, are associated with susceptibility to UC^[25]. Moreover, it is well accepted that IBD patients have a decreased ratio of IL1RN/IL1B in their colonic mucosal tissue^[26].

The variant alleles of two IL1B promoter polymorphisms, IL1B T-31C and IL1B C-511T, have been found to be in almost complete linkage disequilibrium, and the haplotypes encompassing the IL1B T-31C variant conferred higher transcription of IL1B compared to the wild type haplotype^[27].

Four polymorphisms (rs315951, rs315952, rs419598 and rs16944) in the *IL1B* and *IL1RN* genes were analyzed in Mexican Mestizo UC patients. The first 3 single nucleotide polymorphisms (SNPs) are located in the *IL1RN* and the fourth one in the *IL1B* gene. The first two (rs315951 and rs315952) are associated with the risk of developing UC. They found significant increased frequencies of IL1RN6/1TC (rs315952) and RN6/2CC

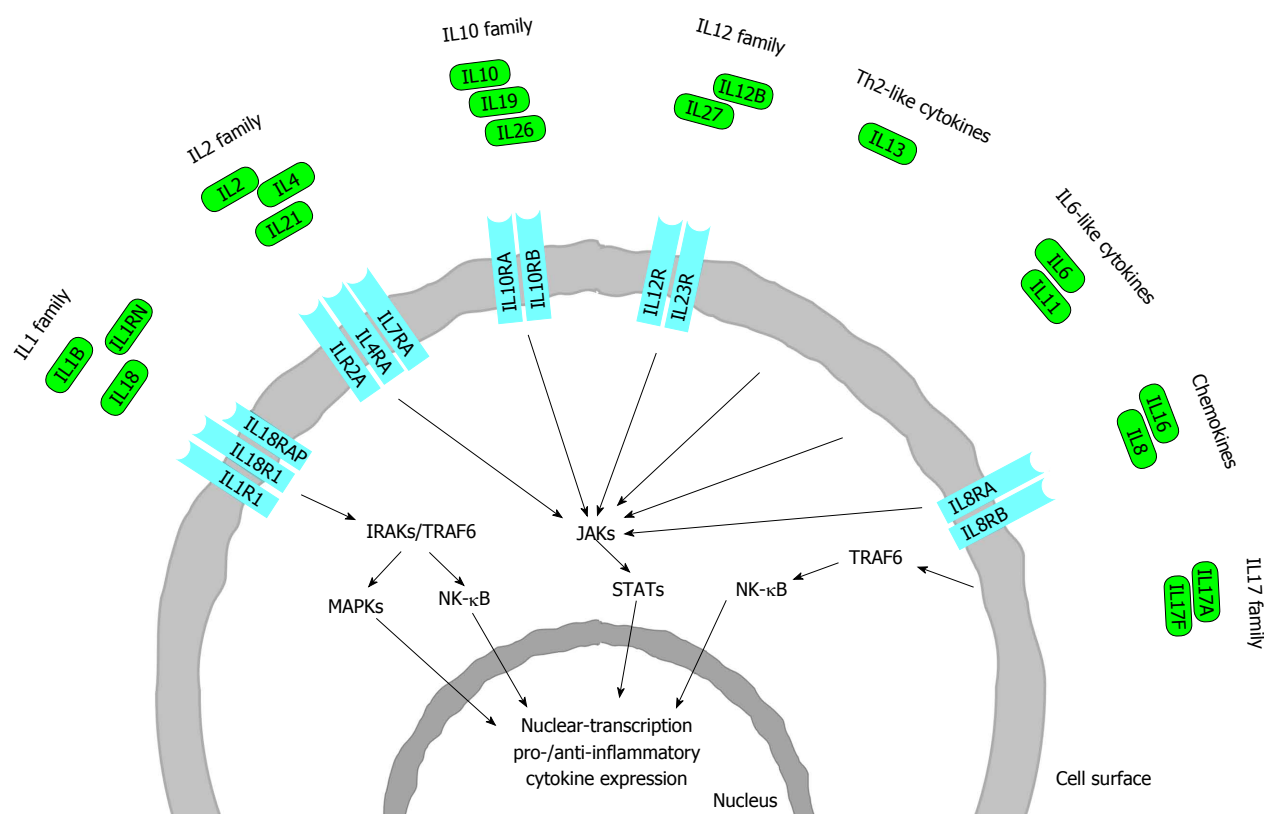


Figure 2 Schematic representation of the interleukin families and receptors involved in the pathogenesis of inflammatory bowel disease. Only those interleukins (IL) and IL receptors (ILR) are shown where studies have demonstrated association between genes/single nucleotide polymorphisms (SNPs) and disease phenotype. ILs are assigned to each family based on sequence homology and receptor chain similarities or functional properties, considerable overlap between these families exists. Polymorphisms in genes encoding ILs and ILRs have been found to be involved in inflammatory bowel disease. Ligand binding initiates intracellular phosphorylation cascades that are mediated by kinases (*i.e.*, IL1 receptor associated kinase (IRAK); mitogen-activated protein kinase (MAPK); Janus kinase (JAK) and TNF receptor associated factor (TRAF), resulting in signal transduction through certain transcription factors [including signal transducers and activators of transcription (STAT); nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B)]. These transcription factors stimulate the expression of a number of proinflammatory and anti-inflammatory cytokine genes involved in inflammatory bowel disease (IBD).

(rs315951), and decreased frequency of IL1B-511 TC (rs16944) genotypes in UC patients. UC patients showed increased frequencies of IL1RN CTC and TCG haplotypes, whereas TTG and CTG haplotypes showed decreased frequency in UC patients. They also found decreased gene expression of IL1RN level in the mucosa from UC patients carrying the rs315951 GG genotype when compared with UC patients with the rs315951 CC genotype^[28].

IL18

One of the main function of IL18 (OMIM 600953) is to promote the production of IFN γ from T and natural killer (NK) cells, particularly in the presence of IL12p70. First it binds to its ligand binding chain the interleukin 18 receptor 1 (OMIM 604494), recruits its coreceptor the IL18 receptor accessory protein (IL18RAP) (OMIM 604509), and the activation of nuclear factor kappa-light-chain-enhancer of activated B cells/mitogen activated protein 8 is initiated. IL18 expression correlates with the activities of CD^[29].

IL18 binding protein (IL18BP, OMIM 604113) is able to prevent the binding of IL18 to its receptor, and

thereby blocks its downstream functional effects. IL18BP has neutralizing isoforms, which have increased levels in the intestinal tissue of active CD patients^[30].

Several polymorphisms were studied in the *IL18* gene: the A105C, the T113G and the C127T in the coding region, and the G-137C, the C-607A and the G-656T in the promoter region. In the Japanese population significant difference was found in the allele frequency of A105C between CD patients and healthy controls. However, there was no association between A105C and UC^[31]. In another Japanese study the G allele at 113 and the T allele at 127 were significantly higher in patients with IBD compared to the control^[32]. In the third Japanese study allele and genotype frequency of G-137C were significantly higher in the proctitis-type UC patients than in controls^[33]. The frequency of haplotype 2 (-607A, -137C), which have lower promoter activity and IFN γ -mRNA level was significantly increased in the proctitis-type patients than in the control group^[33]. Any significant differences in allele or genotype frequencies were observed in the CD group^[33]. The C-607A and the G-137C SNPs in the promoter region were associated with the development of UC but not with CD in Tunisian patients. The

Table 1 Characteristics of cytokines in inflammatory bowel diseases

Family	Cytokine	Receptor	Cytogenetic location	Molecular weight	Cell source	Disease association (IBD)
IL1	IL1A (IL1F1)	IL1R1	2q14	17 kD	Macrophages, monocytes, lymphocytes, keratinocytes, microglia, megakaryocytes, neutrophils, fibroblasts and synovial lining cells	CD, UC
	IL1B (IL1F2)	IL1R1	2q14	17 kD		CD, UC
	IL1RN (IL1F3)	IL1R1 IL1R2	2q14.2	16.1-20 kD	Monocytes, macrophages, fibroblasts, neutrophils, epithelial cells and keratinocytes	UC
	IL18 (IL1F4)	IL18R1 IL18RAP	11q22.2-q22.3	22.3 kD	Macrophages, Kupffer cells, keratinocytes, osteoblasts, astrocytes, and DCs	CD, UC
IL2	IL2	IL2R	4q26-q27	15.5 kD	CD4+, CD8+ activated T cells, DCs, NK and NKT cells	CD, UC
	IL4	IL4R I IL4R II	5q23-q31	15 kD	Th2 cells, basophils, eosinophils, mast cells, NKT and γ/δ T cells	CD
IL10	IL21 IL10	IL21R IL10RA/IL10RB	4q26-q27 1q31-q32	15 kD 18.6 kD	T and NKT cells T and B cells, monocytes, macrophages and DCs	CD, UC CD, UC
	IL19	IL20RA/IL20RB	1q32.2	35-40 kD	Monocytes, keratinocytes, airway epithelial cells and B cells	UC
IL12	IL26 IL12	IL10R2/IL20R1 IL12RB1/IL12RB2	12q15 5q33.3	38 kD IL12A: 35 kD, IL12B: 40 kD	Activated T cells Monocytes, macrophages, neutrophils, microglia, DCs and B cells	CD, UC CD, UC
	IL23 IL27	IL12RB1/IL23R IL27RA/ IL6ST	12q13.13 16p11	19 kD IL27A: 2.8 kD IL27B: 25.4 kD	Macrophages and activated DCs Activated DCs, macrophages, and epithelial cells	CD, UC CD, UC
	IL6-like cytokines	IL6R/IL6ST	7p21-p15	19-26 kD	Endothelial cells, fibroblasts, monocytes/macrophages	CD
	IL17	IL17RA/ IL17RC	6p12	35 kD	Th17, CD8+ T cells, NK cells, NKT cells, γ/δ T cells and neutrophils	UC
Chemokines	IL17F	IL17RA/IL17RC	6p12	44 kD	Th17, CD8+ T cells, NK cells, NKT cells, γ/δ T cells and neutrophils	CD, UC
	IL8	IL8RA/IL8RB	4q13-q21	16 kD	Monocytes, macrophages, neutrophils, lymphocytes, endothelial cells, epithelial cells, fibroblasts, keratinocytes, chondrocytes, synovial cells, and hepatocytes	CD, UC
	IL16	CD4	15q26.3	56 kD	T cells, eosinophils, mast cells, eosinophils, monocytes, DCs, fibroblasts and epithelial cells	CD
Th2-like cytokines	IL13	IL13RA1/IL13RA2	5q31	10 kD	T, NKT, mast cells, basophils and eosinophils	CD, UC

IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative colitis; NK: Natural killer cells; NKT: Natural killer T cells; DCs: Dendritic cells.

-137GG genotype frequency was significantly higher in UC than in controls. Statistically significant association was found between -607AA genotype in UC patients and the distal localization of the lesions^[34]. However the polymorphism G-137C was not found a susceptibility factor for IBD in a German population^[35].

Recent GWAS study^[36] and meta-analysis confirmed the *IL18RAP* region as CD locus^[8]. The rs6708413 G allele is a shared risk locus for CD and Celiac disease^[37]. In individuals homozygous for the risk allele, the genotypes strongly correlate with lower IL18RAP expression which may lead to differential IL18-mediated innate immune responses to infection^[38]. Strong association of rs917997 SNP was demonstrated for both CD and UC^[39]. In a new GWAS study association of CD and IBD with coding variant V527L was found. This rare missense increased

high the risk for CD^[40].

IL2 FAMILY

The IL2 family consists of IL2, IL4, IL7, IL9, IL15 and IL21. This family of cytokines encompasses a group of interleukins which share a common receptor subunit, the "common γ chain", which acts in unison with a subtype specific α -chain to initiate the signaling cascade. This ILs act mainly as growth and proliferation factors for progenitors and mature cells and also have roles in lineage-specific cell differentiation^[13].

IL2

IL2 acts as a T cell growth factor and promotes proliferation and differentiation of NK cells to increase their cy-

tolytic functions. IL2 is essential for the development of Th1, Th2, Treg, and Th17 differentiation^[41].

The IL2 receptor (IL2R) consists of three non-covalently associated proteins: IL2RA (OMIM 147730), IL2RB (OMIM 146710) and IL2RG (OMIM 308380). The α -chain is produced when the T cell is activated by antigen and constitutes the high affinity receptor together with the other two subunits^[42]. The β - and γ -chains form the intermediate affinity IL2 receptor^[43].

Several SNPs (rs6822844, rs13151961, rs13119723 and rs6840978) in the *IL2/IL21* block were analyzed in different populations. In a Dutch cohort the minor alleles of these SNPs were associated with IBD. In UC patients the effect was even stronger. However in the CD subgroup, the rs13119723 SNP was only borderline significant, while only a trend towards association was found for the other SNPs. Testing of all four SNPs in the Italian cohort, the same strong association of the minor alleles in UC was found as in the Dutch cohort. The CD subgroup of the Italian cohort showed only a trend towards association with the same alleles. However in the Jewish population there was any significant association between any of the SNPs and CD. Similarly a North American study showed that these alleles have an influence on IBD. The effect was strongest in the UC subgroup likewise. In the CD subgroup of the North American cohort moderate association with the same alleles was also observed^[44].

IL4

IL4 (OMIM 147780), a pleiotropic cytokine, is the major stimulus of Th2-cell development, which regulates allergic conditions and the protective immune response against helminthes and other extracellular parasites^[45]. There are 2 types of IL4Rs. IL4R Type I binds only IL4 and consists of 2 receptor chains: IL4RA (OMIM 147781) and the common γ c, IL4R Type II binds IL4 and IL13 and consists of the IL4RA and the IL13RA1 chains^[46].

Functional polymorphism in the *IL4* gene promoter C-34T was associated with CD in a British population^[47]. The same polymorphism was tested in a New Zealand population, where no significant difference was observed in the genotype frequencies of controls *vs* CD patients^[48].

IL21

IL21 (OMIM 605384) is a cytokine with potent regulatory effects on cells of the immune system, including NK cells and cytotoxic T cells, which can destroy virus infected and cancerous cells^[49]. In contrast with its anti-cancer effects, IL21 also contributes to inflammation in several disorders, as can be expected for a Th17-related cytokine. The functional receptor of IL21 consists of γ c and the IL21RA (OMIM 605383)^[13].

GWAS provides evidence for 4q27 region *IL2/IL21* association with UC^[44] and CD^[50]. This region contains four genes in strong linkage disequilibrium: *KLA41109-TENR-IL2-IL21*.

IL10 FAMILY

The members of the IL10 cytokine family (IL10, IL19, IL20, IL22, IL24, IL26, IL28, and IL29) are mainly linked through their similar intron-exon structure. This family can be divided into viral and cellular homologs, where this last named group contains the above mentioned ILs^[51].

IL10

IL10 (OMIM 124092) is an anti-inflammatory cytokine, which is produced by monocytes, T cells, B cells, NK cells, macrophages, and dendritic cells. It inhibits both antigen presentation and subsequent release of pro-inflammatory cytokines. Thereby attenuates the activated immune system. The *IL10* gene maps to a cytokine cluster that includes *IL19*, *IL20*, *IL24*, and *IL6* genes. Two IL10RA (OMIM 146933) and two IL10RB (OMIM 123889) chains forms the heterotetrameric IL10 receptor complex. The IL10RB chain is shared with other cytokine receptors^[52].

In a GWAS the rs3024505 showed the most significant association in the combined verification UC samples. Association between rs3024505 and CD was weak. These results suggest that defective IL10 function is central to the pathogenesis of the UC subtype of IBD^[53]. In a latest study from 29 SNPs conferring high genetic susceptibility to CD, the rs3024505 of the *IL10* gene was associated with susceptibility to UC in Australian population^[54]. Similarly to this, the rs3024505 was associated with the risk of UC and CD in a Danish study^[55].

The *IL10* promoter polymorphisms G-1082A, C-819T, and C-592A have been most extensively studied. They are in tight linkage disequilibrium^[56]. Studies on the *IL10* promoter polymorphisms and IBD susceptibility have been controversial^[57-60]. In Spanish population the G-1082A and the C-819T polymorphisms in the *IL10* gene contribute to susceptibility to CD^[61].

In IBD patients in Tunisia the polymorphisms A-627C and G-1117A were analyzed and found as potential factors influencing IBD susceptibility and phenotype. However, no significant variations in genotypes frequencies were found comparing the CD and UC patients^[62].

The A-1082G variant was analyzed in Caucasian population in many studies. It was suggested that G carriers were more susceptible to UC^[63], whereas in another study G carriers were associated with lower UC incidence^[64]. Only two datasets concerning the relationship between A-1082G polymorphism and UC susceptibility in Asian subjects^[65,66] were identified. A meta-analysis showed no association between A-1082G polymorphism and UC susceptibility under any genetic models in overall analysis or in subgroup analysis^[67]. In another study this variant was significantly associated with the colonic localization of the disease in Caucasian CD patients (children) with French-Canadian origin^[68].

IL19

IL19 (OMIM 605687) might promote Th2-cell re-

sponses, because it induces expression of IL4, IL5, IL10, and IL13 by activated T-cells^[69]. IL19 functions as a monomer, binds to a heterodimeric receptor made up of IL20RA (OMIM 605620) and IL20RB (OMIM 605621). This complex also binds to IL20 and IL24^[70].

In GWAS, 14 previously identified UC susceptibility loci were analyzed. Association including the polymorphism rs3024505 in the *IL19* was confirmed^[71].

IL26

Expression of IL26 (OMIM 605679) seems to be restricted to memory T cells, NK cells, and Th17 cells. Thereby it could have proinflammatory effects in CD^[72]. The receptor for IL26 consists of IL10RB and IL20RA chains. Dambacher reported expression of both IL26 receptor subunits IL20RA and IL10RB by several intestinal epithelial cells (IEC) lines in CD^[73].

First time, the rs2870946-G and the rs1558744-A association were described with UC^[74]. Further meta-analysis study confirmed the association of rs1558744-A with UC^[71].

IL12 FAMILY

The IL12 family of cytokines includes IL12, IL23, IL27, IL30 and IL35, which are important mediators of inflammatory diseases. Each member is heterodimeric complex composed of two subunits whose expression is regulated independently and have very different biological activities^[75].

IL12

IL12 (also known as IL12p70) was first described as a NK stimulating factor. It mediates development and maintenance of Th1 cells by inducing production of IFN γ by Th1 and NK cells. IL12 indirectly activates the antimicrobial, antiparasitic, and antitumor activity of macrophages and promotes cytolytic activity of NK cells and lymphokine-activated killer cells^[76]. Reduced production of IL12 impairs Th1 responses and increases susceptibility to infection with intracellular pathogens. IL12 consist of p35 (IL12A, OMIM 161560) and p40 (IL12B, OMIM 161561) subunits, which is shared by IL12 and IL23 cytokines. The IL12 receptor is composed of two subunits, IL12RB1 (OMIM 601604) and IL12RB2 (OMIM 601642), which is homologous to the gp130 subunit^[77,78].

In a German population four *IL12B* SNPs (rs3212227, rs17860508, rs10045431 and rs6887695) were analyzed. The rs6887695 showed association with increased IBD susceptibility, and there was also trend for association with CD and UC. However just a trend was found for association of rs10045431 with UC. A haplotype of all four investigated SNPs showed a trend for association with CD^[79].

From these SNPs the rs6887695 was investigated in Spanish and Japanese population, but with different results. An association was found with CD (rs6887695) and UC susceptibility (rs6887695) in the Japanese cohort, but not to CD susceptibility in the Spanish cohort^[80,81]. Ex-

amining rs1363670 and rs6887695 SNPs in New Zealand population differential effect was found. Carrying the rs1363670 C variant increases risk for CD, while carrying the rs6887695 C variant decreases risk for CD^[82].

In a British cohort an association was found at rs6556416, which encodes a subunit shared by IL12 and IL23. Thus, the Th17 pathway seems as relevant to UC and CD^[83].

IL23

IL23 is a disulfide-linked heterodimer of the p40 (IL12B) and p19 (IL23A, OMIM 605580). IL23 interacts with a receptor composed of IL12RB1 and IL23R chain (IL23R, OMIM 607562)^[78]. IL23 functions in innate and adaptive immunity to regulate Th17 function and expansion^[84]. In addition, this cytokine induces CD8⁺ memory T cells to proliferate and produce IL17. Dysregulation of the IL23/IL17 immune axis has been linked to immunopathology and autoimmune inflammation, like IBD. *IL23R* polymorphisms play role in many autoimmune diseases^[85-87] especially in IBD^[88]. Polymorphisms in the *IL23R* represent one of the strongest associations in CD, and they have also been linked to the pathogenesis of UC^[89].

The *IL23R* gene was identified as a CD susceptibility gene in a North American population. Several independent functional SNPs of the gene and its neighboring region were determined, several were found susceptible to (rs10889677, rs11209032, rs11465804, rs11805303, rs1495965, rs2201841, rs1004819) and the others were protective (rs10489629, rs11209026, rs1343151, rs7517847) against IBD in non-Jewish subjects^[89]. After the primary publication, numerous replication studies have been published these *IL23R* genetic polymorphisms in IBD.

From the susceptibility variants the rs1004819 and rs1495965 were found as important risk factors for CD in Koreans^[90]. Similarly to these results the rs1004819 had the most significant association with CD in Germans, and the another rs10889677, rs2201841, rs11209032 showed increased genotype and allele frequencies comparing the CD cohort to the controls^[91]. Positive association was described of *IL23R* rs10889677 and rs1004819 SNPs with CD in Brazilian population, where the allele frequencies of the patients' group differ significantly from the controls^[92]. Another susceptibility factors were studied in Chinese cohort and the findings showed that rs7530511 and rs11805303 of *IL23R* gene showed positive association with UC susceptibility^[93]. In Jiangsu Han population the rs11805303 was found as a susceptibility polymorphism with UC too^[94].

The protective variants of the *IL23R* gene were analyzed in different populations. Association with rs11209026 and rs7517847 SNPs were confirmed in English subject, where the most significant SNP was the rs7517847^[95]. Similarly in Spanish population the rs7517847 and the rs11209026 showed association with IBD too, the rs7517847 showed the most protective effect against CD and UC^[80,96]. In another study the rs11209026 coding variant was found as a strong protection against CD in German pediatric CD patients^[97].

Five polymorphisms were analyzed in Hungarian IBD population (rs1884444, rs11805303, rs7517847, rs2201841, rs10889677 and rs11209032)^[98]. The rs2201841 and rs10889677 homozygous variants confer risk for the disease, while rs7517847 GG genotype has a protective effect against the development of CD. In Hungarian CD population two *IL23R* gene risk variants the rs2201841 and rs1004819 were found to be a susceptibility factor for CD^[99]. In another study with Hungarian CD patients increased prevalence of the homozygous rs10889677 AA and homozygous rs2201841 CC genotypes were found. The rs10889677 AA genotype was significantly increased in CD patients. The logistic regression analysis showed the AA genotype represents an independent risk factor for the development of CD^[100].

IL27

IL27 is a heterodimeric cytokine consisting of Epstein-Barr virus-induced gene 3 (EBI3, OMIM 605816) and p28 (also known as IL30, OMIM 608273). It binds a unique receptor subunit IL27RA (OMIM 605350), which is associated with gp130 (IL6ST, OMIM 600694), a common chain utilized by IL6 family cytokines. IL27 suppresses Th2 and Th17 differentiation and proliferation^[101,102].

In a Korean population the -A965G SNP was described as a susceptibility factor for IBD^[103]. In a GWAS five new regions were identified near the *IL27* gene associated with early-onset IBD susceptibility (rs8049439, rs2412973, rs1250550, rs4676410 and rs10500264)^[104].

IL6-LIKE CYTOKINES

Cytokines in this family (IL6, IL11, IL27 and IL31) signal through receptors containing gp130 which are commonly referred to as the IL6-like or gp130 utilizing cytokines family^[105]. They show pleiotropic biological activities with immune, hematopoietic, and neural systems^[105].

IL6

IL6 (OMIM 147620) is a multifunctional, pleiotropic cytokine involved in regulation of immune responses, acute-phase responses, hematopoiesis, and inflammation. IL6 signals through a cell-surface type I cytokine receptor complex consisting of the ligand-binding IL6R chain (OMIM 147880), and the shared signal-transducing component IL6ST (also called gp130; OMIM 600694)^[106].

English and Swedish children with CD and *IL6-174* GG genotype were more growth retarded at diagnosis and had higher levels of the IL6-induced inflammatory marker C-reactive protein (CRP) than children with GC or CC genotypes, concluded that *IL6-174* genotype mediates growth failure in CD^[107]. In an Irish cohort from Dublin, significant difference was found in the frequency of *IL6-174* genotypes in the UC group compared with the CD group^[57]. In a Caucasian population from Canada the same polymorphism was analyzed in CD and UC patients. There were significant difference IBD, UC and CD susceptibility, but it has influence on the clinical phe-

notype of CD^[108]. In a Spanish population^[109] homozygous for the *IL6 G-174C* polymorphism showed six-fold higher risk for CD. The GG genotype is associated with a greater production of IL6 compared with GC or CC genotypes^[110].

IL17 FAMILY

This cytokine family is a recently discovered group of cytokines with six members (IL17A, IL17B, IL17C, IL17D, IL17E and IL17F). IL17A was the original member of this family. The others were discovered primarily from the genome sequences within a short time-period (2000-2002), and were sequentially named in the order of discovery^[111-114]. They share the highest amino acid sequence homology and perform distinct biological functions^[115].

IL17

IL17A (OMIM 603149) acts on a variety of cells, which respond by upregulating expression of proinflammatory cytokines, chemokines, and metalloproteases. It is involved in the development of autoimmunity, inflammation, and tumors. IL17E (IL25, OMIM 605658) is an amplifier of Th2 immune responses. IL17F (OMIM 606496) is mainly involved in mucosal host defense mechanisms. The functions of IL17B (OMIM 604627), IL17C (OMIM 604628), and IL17D (OMIM 607587) are still largely elusive. Increased levels of IL17A^[116] and IL17F^[117] have been found in patients with IBD. It inhibits the proliferation of IECs, suggesting it might interfere with the repair mechanism important for the maintenance of the tissue integrity^[118].

The IL17 receptor (IL17R) family includes five members: IL17RA (OMIM 605461), IL17RB (OMIM 605458), IL17RC (OMIM 610925), IL17RD (OMIM 606807), and IL17RE (OMIM 614995)^[119].

In a Japanese UC patients the rs2275913 polymorphism in the *IL17A* gene and the rs763780 in the *IL17F* gene were analyzed. The frequencies of -197A/A and 7488T/T genotypes were found significantly higher in UC patients than in controls^[120]. In a Caucasian (German) population, despite the increased colonic IL17F expression in CD, any significant differences could be found in the frequency of rs763780 on IBD susceptibility^[117].

INTERLEUKINS WITH CHEMOKINE ACTIVITY

This group contains only two ILs, the IL8 and IL16^[13].

IL8

IL8 was identified first as a neutrophil-specific chemotactic factor and later classified as a member of the CXC chemokine family^[121]. Its receptors are: CXCR1 (IL8RA) and CXCR2 (IL8RB)^[122]. IL8 plays crucial role in the chemotaxis and migration of neutrophils, monocytes, lymphocytes, and fibroblasts^[123].

In a Polish population significant association was

found between the genotype frequencies for the heterozygote of the *IL8* T-251A and IBD. When IBD patients were subdivided in UC and CD subgroups this association was also observed. Significant difference was found between the A allele and the UC and CD cases, but not in the summarized IBD group^[124]. In a Chinese population any association was found between the T-251A polymorphism and UC^[125]. They also investigated the role of other polymorphisms of the *IL8* gene and its impact on the level of IL8 in serum. The frequency of the -353A/-251A/+678T haplotype was significantly higher in UC patients than in healthy controls. This haplotype tends to be more common in severe UC patients than in mild to moderate cases^[125].

IL16

IL16 (OMIM 603035) is a proinflammatory cytokine, which inhibits T-cell proliferation, promotes Th1-mediated responses, and reduces Th2-mediated inflammation^[126]. IL16 mediates its biological activity *via* CD4 molecule, which is present on T cells, monocytes, macrophages, and dendritic cells. Patients with CD have elevated levels of IL16^[127].

Regardless of the disease phenotype or the site of intestinal involvement, the T allele and the TT genotype in the *IL16* promoter region T-295C were found significantly increased in German CD cohort, but not in UC patients^[128].

TH2-LIKE CYTOKINES

Cytokines produced during the induction and function of Th2 response include IL4, IL5, IL9, IL13, IL25 (IL17E), IL31, and IL33^[13].

IL13

IL13's (OMIM 147683) receptors are IL13RA1 (OMIM 300119) and IL13RA2 (OMIM 300130), signaling occurs *via* the IL4R complex type II, which consists of IL4RA and IL13RA1. IL13RA2 acts as a decoy receptor of IL13. IL13 activates the same signal transduction pathways as IL4 and induces IgE production, influences eosinophils and cause their prolonged survival, activation, and migration to inflammatory lesions^[129]. IL13 plays an opposite role to IL8. In monocytes and macrophages, it inhibits the secretion of pro-inflammatory mediators such as prostaglandins, reactive oxygen species (ROS) and nitrogen species, TNF α , IL1, -6, -8, and -12^[130].

Presence of the *IL13* -1112 CT (rs1800925) genotypes in a Polish population showed higher risk of IBD as well as UC occurrence. The statistically significant differences in the T-allele distribution were observed in all the investigated groups^[124].

CONCLUSION

In this review we discuss IL and ILR gene polymorphisms which contribute to IBD, CD or UC in different ethnical

population. The cytokine network is highly complex with interactive cascades of gene activation and suppression. Not only the *IL* and *ILR* gene polymorphisms are in relation with IBD pathogenesis but also the downstream signaling components of several ILs (*i.e.*, JAKs, STATs) which could be potential targets of novel treatment strategies. Many IBD loci are also implicated in other immune-mediated disorders, most notably with ankylosing spondylitis and psoriasis^[9]. Since the individual associations may be non-informative, specific combinations of cytokine genotypes might predispose to disease susceptibility or outcome. Therefore, polymorphisms in cytokine genes and receptors should not in all cases be studied strictly in isolation. More complete understanding of the immunopathogenic role of the various ILs in intestinal inflammation will help in the development of more effective novel therapeutic strategies in IBD. Albeit genotyping these interleukin variants are often offer on the palette of several direct-to-costumer companies, their diagnostic or therapeutic clinical use is very limited due to the limited clinical utility and validity of them. Meanwhile, the next generation techniques in combination with the data analysis by systems-biology approach hopefully will contribute to the personalized therapy of the patients in the near future.

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WJG 20th Anniversary Special Issues (3): Inflammatory bowel disease

Trefoil factors in inflammatory bowel disease

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also in protecting mucous epithelia from a variety of insults. This review describes the trefoil factor family and the role of the peptide family in relation to inflammatory bowel disease (IBD), and we summarize the current knowledge of their expression, possible function and potential pharmacological role in IBD.

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Abstract

Inflammatory bowel disease (IBD), which comprises ulcerative colitis and Crohn's disease, is characterized by inflammation of the gastrointestinal tract. The trefoil factors 1, 2, and 3 (TFF1-3) are a family of peptides that play important roles in the protection and repair of epithelial surfaces, including the gastrointestinal tract. TFFs may be involved in IBD pathogenesis and are a potential treatment option. In the present review, we describe the TFF family and their potential role in IBD by summarizing the current knowledge of their expression, possible function and pharmacological role in IBD.

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Key words: Trefoil factors; Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; Inflammation

Core tip: Ulcerative colitis and Crohn's disease are characterized by mucosal inflammation. The trefoil factor (TFF) family consists of three peptides, TFF1, TFF2 and TFF3, and all are widely distributed in the mucous membranes of the gastrointestinal tract. The TFFs facilitate a significant role not only in mucosal repair but

INTRODUCTION

Ulcerative colitis (UC) and Crohn's disease (CD) are the two most common inflammatory bowel diseases (IBDs). The etiologies of both diseases are unknown but are considered to be multifactorial, involving the genetic composition of an individual, the commensal gut flora and the environment^[1].

Studies of the mucosal barrier indicate that trefoil factors (TFF) facilitate a significant role not only in mucosal repair but also in protecting mucous epithelia from a variety of insults in the gastrointestinal tract^[2]. In this respect, repair is essential for preventing inflammation and ulceration. In conjunction with other mechanisms, several products that are primarily secreted from the goblet cells, including TFF and mucins form the innate immune response and first line of defense in the mucus layer. How this fully occurs is still only partly understood^[3].

In mammals, the trefoil factor family consists of three peptides: TFF1, TFF2 and TFF3; all three are widely distributed in the gastrointestinal tract and are present in virtually all mucous membranes. The importance of TFFs in the protection and repair of epithelial surfaces is well established^[4].

TFF1 and TFF3 each have one trefoil domain, while

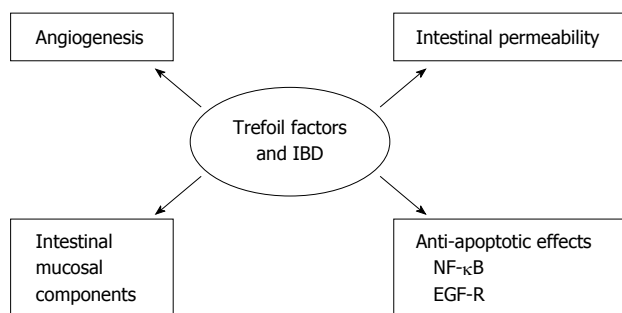


Figure 1 Role of trefoil factors in inflammatory bowel disease. The potential mechanisms involving anti-apoptotic properties, migration and invasion, angiogenesis, and interaction with mucins. IBD: Inflammatory bowel disease; EGF-R: Epidermal growth factor receptor; NF-κB: Nuclear factor-kappa B.

TFF2 has two trefoil domains. The trefoil domain is characterized by a sequence of amino acid residues, in which 6 cysteines are linked by 3 disulphide bonds to form the “trefoil” disulphide loop structure or the clover-like shaped structure^[5]. The resistance of the peptides to proteolytic digestion, acids and thermal degradation seems to be caused by the compact trefoil structure of the peptides^[6,7]. TFF1 and TFF3 only contain one trefoil domain but have a seventh free cysteine, which is essential for the formation of dimers^[6]. It is not clear whether the main part of naturally occurring TFF1 and TFF3 consists of monomers or dimers^[2].

TFF2, formerly known as the Pancreatic Spasmolytic Polypeptide, was the first TFF to be isolated in the early 1980s from a side-fraction obtained during the purification of insulin from porcine pancreas^[7]. The human homologue of TFF2 is produced primarily by mucus neck cells in the body and in antral glands of the stomach, while a small amount is expressed in Brunner’s gland in the duodenum^[8,9]. The cloning of an estrogen-regulated gene from the MCF-7 human breast cancer cell line resulted in the identification of pS2, which is today known as TFF1^[10]. TFF1 is also produced in the stomach by superficial gastric foveolar cells^[11]. It was also discovered that these peptides share a new sequence motif, named the trefoil domain^[5]. A third trefoil factor, TFF3, was identified in 1991 as a rat cDNA sequence^[12] and a human cDNA sequence in 1993^[13] that was initially known as the intestinal trefoil factor (ITF). TFF3 is expressed in the goblet cells of the small and large intestine^[14] and is co-produced and secreted with mucin (MUC)2^[15].

This review describes the TFF family and the role of this family as it relates to IBD and summarizes the current knowledge of their expression, possible function and pharmacological role in IBD.

FUNCTIONAL CHARACTERISTICS OF TFFS

The physiologically relevant functions of TFFs are not clear, and the important question of how TFFs work remains unresolved. Do they work by cross-linking

with mucins, *via* a receptor, or in a completely different way? Data suggest that TFFs may have multiple cellular functions^[16,17]. Below, we describe the potential mechanisms involving anti-apoptotic properties, migration and invasion, angiogenesis, and interaction with mucins (Figure 1).

Anti-apoptotic properties are very important for epithelial restitution, where epithelial cells must migrate over the denuded area of the gut mucosa. In this process, the epithelial cells are vulnerable to apoptosis or anoikis, which is the form of apoptosis that is induced by anchorage-dependent cells detaching from the surrounding extracellular matrix. TFFs have been found to have anti-apoptotic effects in several cell lines^[18,19], and this effect has been supported by the finding that TFF3-deficient mice have increased numbers of apoptotic cells in their colonic crypts^[17]. Furthermore, TFF3 has anti-anoikis effects on intestinal epithelial cells *via* its activation of nuclear factor-kappa B (NF-κB)^[20]. This effect of TFF was dependent on the activation of epidermal growth factor receptor (EGFR) and required TFF3 dimer^[18].

An abnormal distribution or expression of tight junction proteins in gastrointestinal epithelial cells, which causes barrier dysfunction, is thought to be involved in IBD pathogenesis^[21]. The effect of TFF3 on increased intestinal permeability and its association with tight junction proteins was evaluated in an *in vitro* intestinal epithelia barrier model in which colorectal epithelial cells were treated with platelet-activating factor (PAF). The analysis revealed that TFF3 suppressed the PAF-induced down-regulation of the tight junction proteins claudin-1 and zonula occludens-1. These proteins maintain the tight junction’s integrity and intestinal barrier function, and TFF3 thereby decreases mucosal permeability^[22]. TFF3 induces the recovery of tight junction protein changes, which contributes to the TFF3-mediated stabilization and maintenance of intestinal epithelial barrier function. The findings may provide new insight into the protective functions of TFF3 in epithelial cells and demonstrate its potential for treatment of IBD^[22,23].

The proliferative phase of wound healing is characterized by angiogenesis and pro-angiogenic properties, which are dependent on cyclooxygenase-2 and EGFR signalling and have been described for all TFFs^[24].

Alterations in the intestinal mucous components may impair the barrier function of the mucin layer and may be a contributing factor to IBD^[25]. In IBD, the mucin types and expression are affected by several factors. For example, the numbers of mucin-producing goblet cells are reduced in active disease and changes in the thickness and composition of the mucous gel layer may occur^[26]. Although MUC2 is the major colonic mucin, alterations in the composition and concentrations of colonic mucins occur in IBD^[26,27].

Several studies support the hypothesis that TFFs interact with mucins to enhance the mucosal barrier. TFFs and mucins are co-localized in the gut. When TFF3 and mucin were combined, they were more effective in pro-

tecting epithelial cells in an *in vitro* model of epithelial barrier, which indicates a joint effect in mucosal protection^[28]. The TFFs may act differently when coupled with specific mucins, which is supported by the finding that each TFF co-localizes with its own unique mucin type in the ulcer-associated cell lineage (UACL) and normal gastrointestinal mucosa. For example TFF1 couples with MUC5AC, TFF2 couples with MUC6 and TFF3 couples with MUC2^[29].

The combination of mucins and TFFs has been demonstrated to protect cell monolayers against injurious agents by increasing mucus viscosity and decreasing proton permeation^[28,30]. TFF2 in particular has been shown to increase the viscosity and elasticity of porcine gastric mucus and may contribute to a more resilient protective barrier than TFF3. Conversely, TFF1 and TFF3 do not increase the viscosity of mucus but instead form small complexes with the mucins^[31], which may be beneficial in the intestines. TFF1 binds to the von Willebrand Factor C domain of MUC2^[32]. TFF3 was recently reported to form a disulfide-linked heteromer with IgG Fc binding protein, which could contribute to the stability of the mucin network in the mucus layer by interacting with MUC2 mucin^[33].

In addition to the direct stabilization of the surface mucous layer, TFF-mucin interactions have also been shown to promote cellular effects such as cell migration and NO production^[34,35].

TFFS IN EXPERIMENTAL IBD

In animal models of IBD induced by the intrarectal administration of 5% acetic acid, various and conflicting patterns of altered/increased TFF expression have been observed^[36]. A possible explanation for the conflicting results observed in studies exploring the effects of TFFs in the treatment of gastrointestinal damage may be that different TFF forms, dosages and administration routes have been used in colitis models.

In general, the expression pattern of the TFFs in animal models differs from the pattern observed in humans, but the models have been very useful for investigating, for example, the temporospatial expression of TFFs after the induction of damage and the possible use of TFFs for pharmacologic intervention in IBD.

In vivo studies have clearly shown that TFFs have protective and healing effects when given exogenously following either enteral or parenteral administration. This finding suggests that TFFs might be useful in the IBD treatment. In this instance, rodent colitis models have been useful for examining the relationship between intestinal damage and the expression of the TFFs and thus examining the role of exogenously added TFFs in epithelial repair during instances of injured mucosa.

Mashimo *et al.*^[37] showed that mice lacking TFF3 had impaired mucosal healing, with poor epithelial regeneration after injury; those mice died from extensive colitis after oral administration of dextran sulfate sodium (DSS),

an agent that causes mild epithelial injury in wild-type mice. The same was observed following chemotherapy and radiation-induced damage^[38]. In addition, luminal treatment with recombinant TFF3 (rTFF3) restored the capacity for restitution in TFF3-knockout mice exposed to DSS and radiation-induced damage^[37,38].

Although several animal studies have documented the effect of treatment with TFFs, the optimal administration route for treatment with TFFs remains unclear. In animal models of gastric ulceration, both oral and systemic treatments with TFFs are effective for protection, prevention and healing^[39-43]. Subcutaneous infusions of recombinant TFF2 (rTFF2) or porcine TFF2 (pTFF2) decreased acute gastric ulceration damage by 50% without changing the gastric acid secretion^[39,40]. In the gastric ulceration model, both orally and subcutaneously administered TFF2 had an effect; however, both treatments aggravated duodenal ulcerations. After oral administration, pTFF2 is bound to the mucus layer of the stomach and small intestine, but it does not reach the colonic mucosa^[41]. The same dose of pTFF2, given subcutaneously, was superior to oral pTFF2 treatment. When administered orally as a prophylactic treatment, hTFF2 had a protective function in Non-steroidal anti-inflammatory drug (NSAID)-induced damage in rat gastric mucosa^[42], whereas both rTFF2 and rTFF3 prevented ethanol- and indomethacin-induced gastric injury when given up to 2 h before injury. However, following intraperitoneal administration, the rTFF2 treatment had no effect^[43].

The effects of TFFs have also been demonstrated in animal models of intestinal inflammation and damage. Here, the optimal route of administration is unclear, with some studies favoring oral treatment and others favoring systemic treatment^[44-46]. In a DSS-induced experimental colitis model, pre-treatment with subcutaneously or intracolonic administered TFF2, ameliorated the clinical course of this chemically induced colitis, with the luminal route being superior to the parenteral route^[44]. In another study, the distribution profile of subcutaneously and intraperitoneally administered TFF3 was very similar to the intravenous distribution, with a high uptake of tracer in the kidney and gastrointestinal organs. TFF3 availability was slightly faster following *sc* administration than following *ip* administration, and both administration routes would yield comparable pharmacological effects^[45]. The molecular forms of TFFs also seem to play a role. In a DSS colitis model and in a model of colitis induced by the intraperitoneal injection of mitomycin luminal treatment with dimeric TFF3 was effective, whereas treatment with monomeric TFF3 had no effect. It is worth mentioning that a systemic TFF3 monomer treatment intensified the mucosal insults^[47]. In a previous study, the subcutaneous administration of the hTFF1 dimer was proven to be more compelling than the TFF1 monomer^[48].

The DSS model is considered to be suitable for studying acute epithelial damage, but the model lacks the chronic inflammation characteristics of IBD. In colitis models that are more representative of IBD, such as the

dinitrobenzene sulphonic acid model, hTFF2 has shown enhancing effects on colonic epithelial repair and a decrease in local inflammation after luminal application. In addition, endogenous concentrations of TFF2 and TFF3 were increased in the active phase of colitis and reduced to basal levels after hTFF2 treatment^[49].

The chronic production of NO *via* inducible nitric oxide synthase (iNOS) leads to tissue damage and inflammation. In a study involving local intracolonic hTFF2 treatment, the *in vitro* inhibition of NO and iNOS in monocytes was observed, along with a reduction in the levels of the damaging reactive oxygen species and a decrease in colitis. These findings further indicate that TFFs exert a positive effect on mucosal protection^[50].

In combination therapy, TFF3 and EGF act in a synergistic manner to stimulate cell migration *in vitro* and can potentially provide a more effective and safe approach for treating intestinal ulcerations^[51]. This may reduce the degree of colonic injury and may prove to be useful when treating colitis in patients with a disease that is beyond the reach of enema therapy^[46].

Poulsen *et al.*^[52] showed that orally given TFF did not reach the colon. Systematically administered TFF2 and TFF3 bind to the gastric mucosal surface and are transported to the lumen. Whether this occurs in the colon is uncertain, but it seems that less of the systemically administered TFF is present in the colon than in the stomach^[52,53]. The intragastric administration of the TFF1-secreting *Lactococcus lactis* (*L. lactis*) in DSS-induced colitis was followed by the active delivery of TFF at the colonic mucosa. A significant protective effect was observed, which may represent a new therapeutic approach that involves the *in situ* secretion of TFF by orally administered *L. lactis*^[54].

In another recent attempt to improve the application of TFFs, a recombinant adenoviral vector containing the human ITF (*hITF*) gene was constructed and shown both to promote cellular migration in an *in vitro* intestinal wound model and to improve the healing of intestinal mucosal injury^[55].

REGULATION OF TFF EXPRESSION IN IBD

Several studies have shown that the cytokines and transcription factors that are related to the immune system and important in IBD can regulate the expression of TFFs and *vice versa*.

The tumor necrosis factor alpha (TNF- α) triggering of NF- κ B activation is known to be a proinflammatory factor in the pathogenesis of IBD and may contribute to the development of ulcerations. Toll-like receptor-4 (TLR4)/NF- κ B expression is essential for the activation of human intestinal epithelial cells and the subsequent expression of cytokines. Using cell culture studies, it was shown that both TNF- α and NF- κ B induced the down-regulation of TFF3 by repressing transcription and in experimental colitis, the increase in the epithelial expression of NF- κ B coincided with reduced TFF3 expression

during the acute phase^[56].

In a more recent study, the intraperitoneal application of rTFF3 promoted a protective effect against colitis (trinitrobenzene sulphonic acid-induced) and was accompanied by a reduction in TNF- α expression in the colonic endothelium. The protective effect was also paralleled by a reduction in TLR4/NF- κ B expression, indicating that hTFF3 may have therapeutic potential through the inhibition of the TLR4/NF- κ B signaling pathways^[57].

Podolsky *et al.*^[58] investigated the possible linkage between TLR2 that plays a key role in the innate immune system as well as in the digestive system. This possible linkage was studied for TFF3 in a DSS-induced colitis model and for TLR2 and TFF3 in knockout mice models. The oral administration of a TLR2 agonist in TFF3 and TLR2 knockout mice causes anti-apoptotic protection of the TFF3 stress-induces inflammatory intestinal mucosa. Recombinant TFF3 administration decreased morbidity and mortality during acute colonic injury in a TLR2-deficient mice model. These findings imply that TLR2 exerts diverse mucosa-protective properties in different epithelial cell types, critically suppressing mucosal apoptosis and the associated leukocyte influx during acute colitis by regulating TFF3 in goblet cells.

Several studies have indicated that TFF expression may be regulated *via* the EGFR; TFFs and EGF are co-expressed in the UACL cell line^[59]. The transcription of TFF1 is enhanced by EGF^[60], and EGFR activation is required for the auto- and cross-induction of TFFs and for the anti-apoptotic effect of TFF3^[18]. Additionally all TFFs have been shown to cause transient phosphorylation of the EGFR^[61]. However, no binding to the EGFR has been demonstrated, and the mechanisms remain unclear.

In a recent study, it was shown that dietary supplementation with conjugated linoleic acid, which may have anti-inflammatory effects, protected against DSS-induced colitis in a process involving the induction of TFF3^[62].

Overall, multiple studies have investigated, with conflicting results, the relation between TFFs and cytokines as well as the transcription factors related to the immune system. More studies are clearly needed to describe the regulation of TFFs in IBD and thus pave the way for drug development.

TFFS IN CLINICAL STUDIES

Although multiple *in vitro* and animal studies since the discovery of TFF 30 years ago have documented the crucial role of TFFs in the epithelial restitution of the gut, few studies have been performed in IBD patients to investigate the clinical potential of TFF in IBD.

TFF are expressed in several tissues that contain mucus-secreting cells, but they are most markedly expressed in the gastrointestinal tract. At this site, each TFF is co-localized with its unique mucin type. For example, TFF1 is co-localized with MUC5AC, TFF2 is co-localized with MUC6, and TFF3 is co-localized with MUC2^[63,64]. TFF1 and TFF2 are primarily located in the stomach^[8,11], where-

as TFF3 is predominantly present in the mucous cells of the small and large intestine^[14]. Several studies have documented the supportive and protective functions of TFFs in the human gastrointestinal tract. Those studies have shown the up-regulated expression of all three TFFs at the site of mucosal damage in IBD^[65,66], peptic ulcer^[67] and the neoexpression of TFF1 in UC with histologically severe disease^[68].

The UACL, which occurs at sites of chronic gastrointestinal ulceration including IBD expresses a number of peptides that have been implicated in the repair of damaged mucosa, such as the TFFs^[13,69]. In small intestinal Crohn's disease, TFF2 mRNA is expressed in the acinar and proximal duct cells, while TFF1 mRNA and peptide are found in the distal duct cells and in the surface cells^[65]. As in normal gastrointestinal mucosa, the co-localization of specific TFFs and mucins is observed in IBD^[29,68].

The co-localization of TFF3 with DMBT1 in IBD, which has been proposed to have a role in cell differentiation and growth, indicates that DMBT1-TFF3 interactions may play a role in IBD^[70].

Quantitative measurements of TFFs have been important tools for elucidating the biological functions of the peptides and exploring their role as biomarkers for IBD. Larger than normal serum concentrations of TFF2 and TFF3, *i.e.*, 2000 to 10000 and 140 to 500 times higher, respectively, have been measured in bowel discharges^[71]. All three TFFs are present in sera from healthy individuals^[72]; in line with immunohistochemical studies showing increased expression in IBD, the sera concentrations of the peptides were also elevated in IBD patients^[73-75]. The TFF3 concentrations were significantly higher in UC patients and their levels correlated with the clinical and biochemical parameters of disease activity.

Because of large biological variations, measurements of TFFs are not useful as clinical biomarkers for disease activity in CD and UC^[72,75]. However, quantitative measurements may still be important and should be included in continued research in the area.

In clinical studies TFF peptides are considered promising for the treatment of inflammatory conditions of mucous membranes. In IBD the effect of TFF3 enemas, given in combination with oral mesalazine in patients with mild-to-moderate left-sided UC, have been tested. UC patients were given a total daily dose of 750 mg of dimeric rTFF3 in 75 mL enemas (dosage concentration of 10 mg/mL), similar to the lumenally administered dose used in animal models of gut injury that has proven effective. The TFF3 enema was well tolerated, but in this first human study no additional benefit of TFF3 treatment was detected compared to the effect of 5-aminosalicylic acid treatment alone^[76]. One possible explanation may be the rapid decay of TFFs observed in the colon^[71]. In future trials, the systemic route should be explored.

CONCLUSION

Since the discovery of the TFFs, a number of animal studies and studies on UC and CD patients have shown

that the peptides are linked to inflammatory conditions in the gut. Although a significant role in mucosal protection and repair has been established for the TFFs, the full knowledge of their biological functions in IBD remains elusive. The quantitative measurements of the peptides in patients with IBD have been less promising, due to large inter-individual variations, and their future use as biomarkers in IBD is uncertain. Future studies are needed to show whether the peptides have a potential as a novel therapeutic in IBD. Additionally further identification of the regulatory mechanisms that can affect TFF expression may aid in the development of new drugs for treating IBD.

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WJG 20th Anniversary Special Issues (3): Inflammatory bowel disease

Biomarkers in inflammatory bowel diseases: Current status and proteomics identification strategies

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Abstract

Unambiguous diagnosis of the two main forms of inflammatory bowel diseases (IBD): Ulcerative colitis (UC) and Crohn's disease (CD), represents a challenge in the early stages of the diseases. The diagnosis may be established several years after the debut of symptoms. Hence, protein biomarkers for early and accurate diagnostic could help clinicians improve treatment of the individual patients. Moreover, the biomarkers could aid physicians to predict disease courses and in this way, identify patients in need of intensive treatment. Patients with low risk of disease flares may avoid treatment with medications with the concomitant risk of adverse events. In addition, identification of disease and course specific biomarker profiles can be used to identify biological pathways involved in the disease development and treatment. Knowledge of disease mechanisms in general can lead to improved future development of preventive and treatment strategies. Thus, the clinical use of a panel of biomarkers represents a diagnostic

and prognostic tool of potentially great value. The technological development in recent years within proteomic research (determination and quantification of the complete protein content) has made the discovery of novel biomarkers feasible. Several IBD-associated protein biomarkers are known, but none have been successfully implemented in daily use to distinguish CD and UC patients. The intestinal tissue remains an obvious place to search for novel biomarkers, which blood, urine or stool later can be screened for. When considering the protein complexity encountered in intestinal biopsy samples and the recent development within the field of mass spectrometry driven quantitative proteomics, a more thorough and accurate biomarker discovery endeavor could today be performed than ever before. In this review, we report the current status of the proteomics IBD biomarkers and discuss various emerging proteomic strategies for identifying and characterizing novel biomarkers, as well as suggesting future targets for analysis.

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Key words: Inflammatory bowel disease; Biomarker; Proteomics; Citrullination; Ulcerative colitis; Crohn's disease; Posttranslational modification

Core tip: Establishing the correct diagnose of Crohn's disease and ulcerative colitis (UC) patients remains troublesome, and correct and early medication is critical. No reliable biomarkers have been implemented in clinical usage, to distinguish between Crohn's disease patients and UC patients. Considering the protein complexity encountered in intestinal biopsy samples and the recent development within the field of quantitative proteomics, submitting the intestinal mucosa to a more thorough analysis has the potential to reveal new biomarkers.

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INTRODUCTION

Inflammatory bowel diseases (IBD) are chronic gastrointestinal disorders. The two most common forms of IBD are Crohn's disease (CD) and ulcerative colitis (UC). Both disorders have great impact on the life quality of the affected individuals and for society, measured on lost labor and expenses to the health care system. Furthermore, new epidemiological data published in 2013 found that the incidence and prevalence of the diseases are still increasing^[1]. The etiologies of CD and UC remain unclear, but involve a complex interplay between genetic and environmental factors^[2-7]. The diagnosis can be delayed several years and may be difficult to make even for trained physicians, as no biomarkers or commercial tests capable of discriminating CD from UC patients have been implemented in clinical use^[8-10]. Furthermore, an early and accurate diagnosis of IBD-patients is crucial, as *e.g.*, CD patients with extensive and deep ulcerations have a 5-fold higher risk of requiring colectomy compared to CD patients without extensive and deep ulcerations^[11]. From 357 CD patients analyzed with computed tomography enterography, penetrating disease was found in 21% of the patients and extraintestinal manifestations in 19%^[12,13]. Hence, there is a need for reliable and usable biomarkers for the early and better diagnosis and prognosis of the IBD diseases^[4,8,14-18].

GENOMIC, TRANSCRIPTOMIC AND PROTEOMIC BIOMARKERS

In 2001, an NIH group defined a biomarker as "A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacologic responses to a therapeutic intervention."^[19], usable for diagnostics, monitoring disease prognosis and disease monitoring and prediction. The human genome contains the code for the expressed gene products, including the proteins. Proteins function as the building blocks of the human cells and tissue, and are responsible for the majority of the biological functions^[20]. Proteins, therefore, represent an obvious target for biomarker discovery studies. The human genome comprises approximately 20000 protein coding genes^[21]. During protein synthesis, the DNA code is first transcribed into different RNA transcripts. Each gene can give rise to several RNA transcripts resulting in a total of roughly 100000 different RNA transcripts^[22-24] (Figure 1), which in turn are translated into 100000 different proteins. After

translation, most proteins are covalently modified at least once^[25], and the final mature protein products are termed proteoforms. These so-called posttranslational modifications (PTMs) are often crucial to the correct physiological function of the given protein, and can determine activity state, localization, turnover and interaction with other proteins and substrates^[23,25,26]. More than 200 distinct biologically relevant PTMs have been identified^[27], so each RNA transcript can be more than 200 different proteoforms. The PTMs increases the complexity and diversity of the proteins tremendously (Figure 1). As a result, it is estimated that the human body contains more than one million different proteoforms^[23], which constitutes the human proteome (all expressed proteins).

When searching for biomarkers, it is possible to analyze the target sample on the DNA level, the RNA transcript level or the protein level. Techniques for studying an organisms DNA code (genome) or RNA transcripts (transcriptome) have the advantage that entire genomes and transcriptomes can be sequenced and studied with great sensitivity, precision and coverage, and a number of biomarkers have been found for various diseases. Using genomic sequencing techniques, several CD and UC loci have been known for more than a decade, and the studies have greatly increased our knowledge of the IBDs^[22,28,29]. Several cellular IBD-pathways have been identified, including pathways involved in barrier function, epithelial restitution, microbial defense, immune regulation, reactive oxygen species generation, autophagy, and finally various stress and metabolic pathways associated with cellular homeostasis, reviewed by Khor *et al.*^[3]. However, as mentioned no IBD biomarkers capable of differentiating CD from UC have been implemented in daily clinical usage, and the impact of the genomic studies on the treatment and diagnosis of the IBDs has been questioned^[30-32].

Proteins represent an obvious target for biomarker discovery studies, and as PTMs dramatically increases the diversity and in many cases function of the mature proteins, they represent a promising area for IBD biomarker studies. PTMs are introduced after translation of the RNA transcripts (Figure 1), hence analyzing DNA and RNA transcripts does not directly provide information about the PTMs. A key technique capable of measuring absolute and relative protein quantification in complex protein mixtures in a high-throughput manner, as well as identify several PTMs, is bottom-up mass spectrometry (MS) based proteomics^[24,33]. Proteomics is the large-scale identification of proteins, and can often cover the study of all expressed proteins by an organism (the proteome). The bottom-up MS strategy is based on measuring the mass-to-charge ratios (m/z) of peptides derived from proteins which have been enzymatically cleaved into minor peptides. From the measured m/z 's the molecular weight of the intact peptides can be calculated^[25]. In addition to calculating the intact masses, the peptides are collided with an inert gas which fragments the peptides, and the fragment m/z 's are measured. The proteins in the sample are subsequently identified by searching the

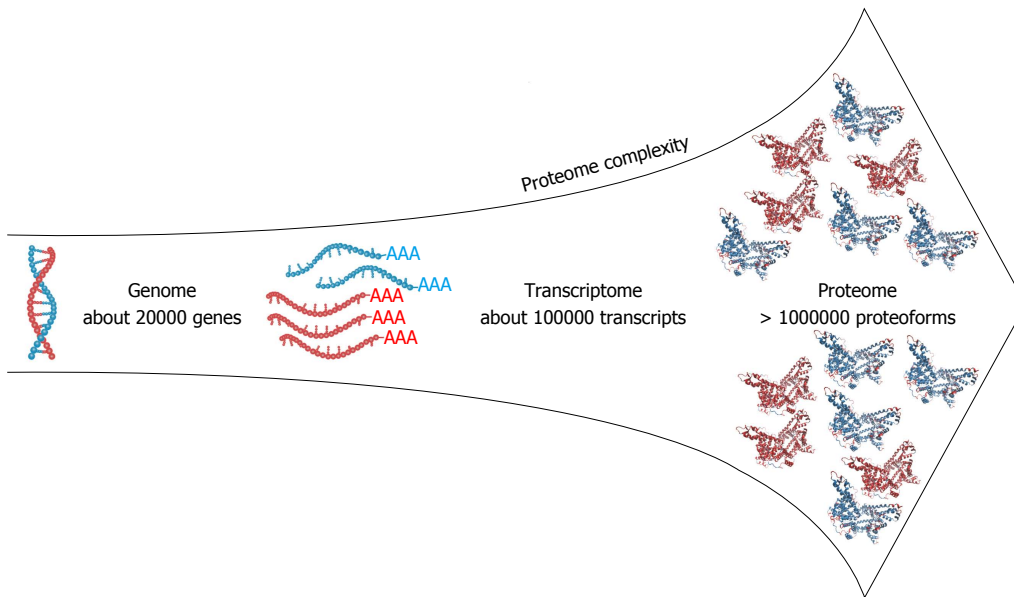


Figure 1 Major increase is encountered in the proteome complexity, from genes to RNA transcripts and finally to the mature, often posttranslational modification modified, proteins (proteofoms).

peptide masses and fragment m/z 's against an *in silico* generated database, inferred from a reference database of protein sequences. By matching the *in silico* calculated peptide masses and fragment m/z 's to the measured, the peptides and hence the proteins, are identified. For a more thorough description, we refer to the review by Steen *et al.*^[34]. The process can be performed in a quantitative manner to allow for relative or absolute quantitation of the proteins, using different strategies^[34]. MS can in this way be used to identify proteins, as well as PTMs that change the molecular weight of the protein and can provide the amino acid position of the modification^[25]. Previously, proteomics has been limited mainly by the speed and sensitivity of the mass spectrometers. However, recent development within the field of MS has allowed for the identification of nearly all expressed proteins of complex organisms, such as yeast, within a few hours of measuring time, identifying and quantifying several thousand proteins^[33,35]. When considering the protein complexity encountered in the human intestinal tissue, an obvious place to search for biomarkers, and the recent development in the field of MS, a thorough analysis of PTMs and protein abundances in healthy and diseased state could be conducted. Biomarkers found in the intestine could then be searched for in more easily obtained sample material, such as blood or stool^[6,10,31,36-39]. Antibodies to identified biomarkers for CD and UC found by proteomics can be generated for development of immunoassays and immunohistochemistry for evaluating the markers clinical use in routine tests less expensive than sequencing genomes, transcriptomes or MS driven proteomics.

This review reports known biomarkers for the IBDs, but will focus on the newly identified proteomics biomarkers and emerging proteomics strategies for identifying and characterizing novel IBD biomarkers.

DIAGNOSIS OF INFLAMMATORY BOWEL DISEASE AND KNOWN BIOMARKERS

Numerous biomarkers are known and used for the IBDs (Table 1); however, no single biomarker is able to diagnose IBD or to distinguish CD from UC patients with a high specificity and sensitivity^[8-10,14]. CD is characterized by chronic inflammation in any part of the gastrointestinal tract. Most commonly the terminal ileum or the perianal region are inflamed, and in a non-continuous manner. Histologically, CD shows thickened submucosa, transmural inflammation, fissuring ulceration and non-caseating granulomas. UC, on the other hand, is characterized by inflammation limited to the colon, spreading continuously from the rectum and various distance proximal, and histology shows superficial inflammatory changes limited to the mucosa and submucosa with inflammation of crypts (cryptitis) and crypt abscesses^[3]. There is currently no single "gold standard" diagnostic test or examination to differentiate CD and UC. Instead, diagnosis is based on a combination of symptoms, clinical examinations, laboratory findings, radiology, and endoscopy with histology, which also is used to assess severity and to predict the outcome of disease. Even when the tests are performed by expert clinicians they can result in diagnostic uncertainty^[10,14,15,17,40]. This section will report some of the biomarkers commonly used to diagnose IBD. For a review of additional IBD biomarkers we refer to the work of Iskandar *et al.*^[41].

Antibodies and serum biomarkers

The two best-studied serological markers in IBD patients are anti-*Saccharomyces cerevisiae* antibodies (ASCA) and anti-neutrophil cytoplasmic antibody (ANCA)^[41].

ASCA is an antibody with affinity for antigens in the

Table 1 Known common inflammatory bowel disease biomarkers

Biomarker	Specificity	Usability
Serum biomarkers		
ASCA	39%-79% of CD patients positive, 5%-15% UC patients ^[41-43]	14%-18% of controls tested positive, limiting the diagnostic value ^[44]
pANCA	20%-85% of UC patients positive, 2%-28% of the CD patients ^[41,42,45]	32% of controls tested positive, limiting the diagnostic value ^[44]
CRP	Marker for acute inflammation	Cannot differentiate CD from UC. However, usable for monitoring disease state ^[48-50]
Fecal biomarkers		
Calprotectin	Sensitive marker for intestinal inflammation ^[8,17,40]	Cannot differentiate CD from UC. Used to monitor disease state ^[17]
Lactoferrin	Can distinguish active IBD from inactive IBD and irritable bowel syndrome ^[60]	Unspecific for CD and UC. However, usable for monitoring disease state ^[60]

ASCA: Anti-*Saccharomyces cerevisiae* antibodies; ANCA: Anti-neutrophil cytoplasmic antibody; IBD: Inflammatory bowel diseases; UC: Ulcerative colitis; CD: Crohn's disease; CRP: C-reactive protein.

cell wall of the yeast *Saccharomyces cerevisiae*. In comparison to UC patients, CD patients are often positive for ASCA (Table 1)^[41-43]. However, a substantial amount of healthy controls are also positive for ASCA positive^[44], indicating that specificity and sensitivity for CD patients are relatively low; limiting the diagnostic value of the marker in differentiating CD from UC.

ANCAs are antibodies with affinity for neutrophil granules. The antibodies have been found in a variety of immune conditions, including Wegener's granulomatosis and rheumatoid arthritis (RA)^[4]. When staining for ANCA, different patterns have been observed for UC and CD patients using immunofluorescence microscopy (Table 1), and mainly UC patients display perinuclear ANCA (pANCA) staining compared to CD patients^[41,42,45]. Nonetheless, like the case of ASCA, a substantial amount of healthy controls are pANCA positive^[44].

Lastly, C-reactive protein (CRP) is one of several proteins that increase in serum upon acute phase IBD. CRP is almost exclusively produced in the liver, upon stimulation by interleukin (IL)-6, tumor necrosis factor (TNF)-alpha and IL-1-beta produced at the site of inflammation. As such, an increased CRP-level is a marker for inflammation, but is not specific for CD or UC^[8,40,46,47]. In some cases, but far from always, CD is associated with a strong CRP serum increase, whereas UC usually only results in a modest response. However, the difference insufficient to differentiate CD patients from UC patients^[48-50], and the reason for the different responses remains to be thoroughly accounted for^[40].

Other serum biomarkers used include white blood cell count, platelets, and albumin, which are all non-specific for IBD and can be seen in inflammatory diseases and cell stress^[40]. More CD serologic markers are described in

the review by Tamboli *et al.*^[51].

Fecal biomarkers

Stools are in direct contact with the inflamed intestinal area and site for the gut microbiome, both from which potential biomarkers are likely to originate. This is in contrast to serum biomarkers, which could increase on account of a variety of conditions, making stools an obvious place to search for biomarkers^[40]. Fecal markers are especially useful for the diagnosis of CD patients, where the inflammation is patchy, may affect any part of the gastrointestinal tract, and therefore might be missed by colonoscopy^[52]. The host-microbe interactions have been recognized as central for understanding human physiological diversity, and the human microbiome project has been launched to unravel the medical significance of the human microbiome^[53]. Several studies have identified certain bacterial groups which are more abundant (*Enterobacteriaceae*, *Ruminococcus gnavus*, and *Desulfovibrio*) or less abundant (*Faecalibacterium prausnitzii*, *Lachnospiraceae*, and *Akkermansia*) in IBD^[16], implicating that the host-microbe interaction might be involved, reviewed by Rosenstiel^[54]. Novel biomarkers with high sensitivity and specificity may, therefore, be identified from stools.

The two most commonly used fecal markers for IBD screening are calprotectin and lactoferrin (Table 1)^[8]. Calprotectin is a calcium- and zinc-binding protein occurring in large amounts in neutrophil granulocytes, where it accounts for 5% of the proteins. It is a very stable marker and is resistant to colonic bacterial degradation, and can be stored at room temperature for more than a week^[55]. The concentration of fecal calprotectin is proportional to the neutrophil cell infiltrate in the bowel mucosa, and it is a very sensitive marker for intestinal inflammation^[8,17,40]. However, calprotectin is not a specific marker for CD or UC, and increased levels can also be found with neoplasia, other forms of IBD, infections, and polyps^[17], as well as with use of non-steroidal anti-inflammatory drugs, increasing age^[56] and upper gastrointestinal disease, such as small bowel bacterial overgrowth^[57].

Lactoferrin is an iron-binding glycoprotein expressed by activated neutrophils^[58]. During inflammation, lactoferrin is released by the injured tissue and has been found to modulate inflammation and act in the defense against infections as a part of the innate immune system^[59]. It is resistant to degradation and proteolysis, and unaffected by freeze thaw cycles, making it a useful biomarker^[17]. As such, it is an ideal marker for intestinal inflammation. However, like calprotein it is unspecific for CD and UC, but can distinguish active IBD from inactive IBD and irritable bowel syndrome^[60]. Several studies report similar performance of calprotectin and lactoferrin tests^[6,60-64], and neither can be used to differentiate CD from UC with a high sensitivity and specificity.

To sum up, no reliable biomarkers exist usable as a single "gold standard". Therefore, to establish a diagnosis, histological examination of biopsies from the terminal ileum and colon is typically used in combination with

patient disease history and one or more of the above mentioned markers^[17,65]. Hence, much effort is invested in analyzing the IBDs using various strategies, to identify usable biomarkers and explain the disease etiologies.

KNOWN PROTEOMICS BIOMARKERS FOR INFLAMMATORY BOWEL DISEASE

Proteomics studies can be performed in a discovery-based manner, where relative protein abundance levels between two or more samples are detected, and PTMs can be identified. Recent development of proteomics platforms has brought the technology to the point where several thousand proteins can be identified and (relatively) quantified in a single analysis or a subset by targeted approaches^[6,10,31,36-39]. As inflammation takes place in the intestine, the gut-tissue represents an obvious place to look for novel biomarkers, which afterwards may be searched for in for example feces and blood and used as a disease marker. Several proteomic studies have successfully been aimed at identifying IBD biomarkers to investigate disease etiologies and aid in establishing the correct diagnose of UC and CD patients (Table 2). However, until now none of the identified biomarkers have been implemented in daily use^[15].

The first group to publish a discovery-based proteomics study of the IBDs was Barceló-Batllori *et al.*^[66] in 2002. The aim of the study was to identify potential cytokine regulated proteins in colon epithelial cells isolated from IBD patients, which might be involved in the pathogenesis of IBDs. Human adenocarcinoma cells were *in vitro* exposed to known cytokines expressed in IBD, namely interferon-gamma, IL-1-beta and IL-6 (TNF-alpha was excluded as it is known to induce apoptosis in such cells). Using proteomics, the protein profiles of the cells were analyzed before and after exposure to the cytokines. All proteins from the cells were first separated using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). By staining all protein in the gels, different samples (gels) can be compared in terms of protein abundance based on the staining intensities, and differentiating protein spots can be visually identified. Spots of interest were cut from the gel with a knife and the proteins were enzymatically digested to specific peptides using the protease trypsin (in-gel digestion). The digestion of proteins is an essential step for protein identification, as no MS technique currently exist that can identify thousands of intact proteins in a complex sample in a high throughput manner. This is only possible when using digested proteins (peptides). The proteins were identified based on the peptides using MS, with the technique called matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) MS (Figure 2A). MALDI-TOF MS is a sensitive technique, but it involves placing a few drops of the sample on a plate which is left to dry prior to analysis. During analysis a laser is used to evaporate small spots from the dried droplet and ions in the produced gas are

analyzed by MS. In the study, several cytokine regulated proteins were identified. Subsequently, human epithelial cells were isolated from UC patients and CD patients. Based on the findings, the samples were analyzed for the enzyme indoleamine-2,3-dioxygenase using antibodies by western blotting. The group found an overabundance of the enzyme indoleamine-2,3-dioxygenase in CD and UC compared to normal mucosa, hypothesizing an involvement of the Kynurenine pathway of tryptophan metabolism in the IBDs. Indoleamine-2,3-dioxygenase activity has furthermore been found to be essential in dendritic cells to induce co-cultured T cell apoptosis^[66].

When analyzing protein spots cut from gels the MALDI-TOF MS method is applicable, but the analysis of an entire 2D-PAGE gel is unfeasible, due to the commonly several thousand detectable spots. The technique is therefore less suitable for high-throughput identification of many thousand proteins. Therefore, when analyzing digested 2D-PAGE gels one usually only investigates changing protein spots and omits any information regarding non-changing protein spots. Information regarding non-changing proteins might prove equally important as changing proteins for studies seeking to describe disease etiologies. However, for biomarker studies 2D-PAGE strategies represent a feasible and proven way of identifying biomarker candidates. MALDI-TOF MS can also be conducted using intact proteins without prior enzymatic protein digestion. A variant of MALDI-TOF MS is to spot the protein mixture on a modified surface, to which the intact proteins bind and subsequently the intact masses of the proteins can be obtained by MS. This technique is called surface-enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-TOF MS) (Figure 2A). However, when studying intact proteins using MALDI-TOF MS or SELDI-TOF MS, one usually does not obtain identification of the detected signals.

Electrospray ionization (ESI) remains the only MS technique for identifying and quantifying several thousands of proteins in a high-throughput manner (Figure 2B). ESI involves spraying the digested proteins directly into the MS. By incorporating liquid chromatography (LC) with columns prior to the ESI process, the peptides can be separated and sequentially eluted over several hours. This gives the MS systems enough time to analyze a large proportion of the eluted peptides which subsequently can be identified. In this way, large-scale proteomic studies can be performed in a high-throughput manner using ESI LC-MS. These studies yield (relative) quantitative information of thousands of identified proteins in a single experiment, and thus might provide better information for explaining disease etiologies. In 2004, Hardwidge *et al.*^[67] published such a study, which was the first large scale proteomic analysis of a human cellular response to a pathogen. Discovery-based proteomics was applied to investigate the protein profiles (cellular response) of human Caco-2 intestinal epithelia cells before and after infection with *E. coli*. The group did not work directly with IBD, but the results are applicable to the diseases,

Table 2 Proteomics biomarker candidate studies and main findings

Ref.	Sample	Analysis	Findings and perspectives
Barcelo-Batllo <i>et al</i> ^[66] , 2002	<i>In vitro</i> colon epithelial cells and purified epithelial cells from UC and CD patients	2D-PAGE protein quantitation, and in-gel digestion and MALDI-TOF MS and Western blot protein identification	The enzyme indoleamine-2,3-dioxygenase was more abundant in cells from CD and UC patients compared to normal mucosa. Tryptophan and arginine metabolism may play a role in the IBDs
Hardwidge <i>et al</i> ^[67] , 2004	Human Caco-2 intestinal epithelia cells before and after infection with <i>E. coli</i>	ESI LC-MS protein identification and quantitation, Western blot verification	125 proteins more abundant and 139 proteins less abundant after infection, some related to innate immune responses. These proteins might be relevant to look for in future biomarker studies
Hsieh <i>et al</i> ^[68] , 2006	Colonic biopsies from UC, nonspecific infectious colitis patients and controls	2D-PAGE protein quantitation, and in-gel digestion and MALDI-TOF MS protein identification	6 proteins were found to be more abundant in UC and 13 less abundant. The result indicates that mitochondrial dysfunction might be involved in UC the etiology. Four biomarker candidates were identified, however, they require validation
Shkoda <i>et al</i> ^[69] , 2007	Intestinal tissue cells purified from patients suffering from CD, UC, and colon cancer	2D-PAGE protein quantitation, and in-gel digestion and MALDI-TOF MS and Western blot identification	Proteins associated with signal transduction, stress response and energy metabolism were differently abundant in inflamed and non-inflamed tissue. 32% of the differentially regulated proteins were involved in energy metabolism
Meuwis <i>et al</i> ^[10] , 2007	Serum from UC and CD patients	SELDI-TOF MS m/z signal profiling, MALDI-TOF MS and Western blot protein identification	Successful in differentiating CD from UC patients with a sensitivity of 85% and a specificity of 95% from several m/z signals. Four biomarker candidates were identified, all known acute inflammatory markers, limiting the diagnostic value. However, the feasibility of serum biomarker studies was demonstrated
Nanni <i>et al</i> ^[71] , 2007	Serum from UC, CD patients and healthy controls	Solid-phase bulk protein extraction, MALDI-TOF MS signal profiling	Able to separate the three groups with 97% prediction results. The signals were not identified, but the feasibility of serum biomarker studies was demonstrated
Meuwis <i>et al</i> ^[70] , 2008	Serum from responding and non-responding CD patients to infliximab	SELDI-TOF MS signal profiling, MALDI-TOF MS, Western blot and ELISA protein identification	Able to predict responders with a sensitivity of 79% and a specificity of 80%. Increased amount of PF4 was associated with non-response to infliximab with MS but not ELISA, so usability of PF4 as a biomarker seems limited
Nanni <i>et al</i> ^[72] , 2009	Intestinal epithelial cells from CD patients and healthy controls	1D-PAGE and in-gel digestion, ESI LC-MS protein identification and quantitation	Proteins more abundant in CD patients include several proteins involved in inflammation processes, and less abundant include Annexin A1, involved in the anti-inflammatory action. Follow-up research is required to assess the feasibility of the biomarker candidates
Hatsugai <i>et al</i> ^[73] , 2010	Peripheral blood mononuclear cells from UC and CD patients, and healthy controls	2D-PAGE quantitation, and in-gel digestion and MALDI-TOF MS protein identification	Successfully discriminated UC from CD based on seven differently present proteins, all associated with inflammation oxidation/reduction, the cytoskeleton, endocytotic trafficking and transcription. The biomarker candidates require validation using a larger number of patients, but seems promising
M'Koma <i>et al</i> ^[74] , 2011	Mucosal and submucosal layers of samples originating from CC and UC patients	MALDI-TOF MS m/z signal characterization, no protein identification	Five m/z signals were detected in the submucosal layer, which could separate the two groups with an accuracy of 75 percent. The signals needs to be identified, however, the disease groups can be separated on basis of the mucosal and submucosal profiles
Presley <i>et al</i> ^[75] , 2012	Microbes and human proteins at the intestinal mucosal-luminal interface from CD and UC patients, and healthy controls	Oligonucleotide ribosomal RNA fingerprinting, SELDI-TOF MS and MALDI-TOF MS identification	35% of the detected bacterial phylotypes were present in different amounts in the diseases, indicating the involvement of host-microbe interactions in IBD. The microbiome might prove useful as a target for therapy
Han <i>et al</i> ^[14] , 2013	Colonic tissue biopsies of Korean IBD patients	ESI LC-MS protein identification with label-free quantitation	27 potential biomarkers were identified for UC, 37 biomarkers for CD and 11 proteins commonly associated with IBD. Three novel biomarkers were identified for active CD: Bone marrow proteoglycan, L-plastin and proteasome activator subunit 1. The biomarker candidates require validation, but might prove feasible as new diagnostic and therapeutic targets
Seeley <i>et al</i> ^[76] , 2013	Histological tissue layers from UC and CC patients	MALDI-TOF MS m/z signal characterization, no protein identification	114 different m/z signals were found to be different between the two groups. The signals remain unidentified
Gazouli <i>et al</i> ^[77] , 2013	Serum samples from responding and non-responding CD patients to infliximab treatment	2D-PAGE quantitation, and in-gel digestion and MALDI-TOF MS protein identification	15 differently abundant proteins between responders and non-responders to infliximab were identified. The biomarker candidates require further validation

IBD: Inflammatory bowel diseases; UC: Ulcerative colitis; CD: Crohn's disease; MALDI-TOF MS: Matrix-assisted laser desorption-ionization time-of-flight mass spectrometry; 2D-PAGE: Two-dimensional polyacrylamide gel electrophoresis; ESI: Electrospray ionization; SELDI-TOF MS: Surface-enhanced laser desorption/ionization time of flight mass spectrometry; LC: Liquid chromatography.

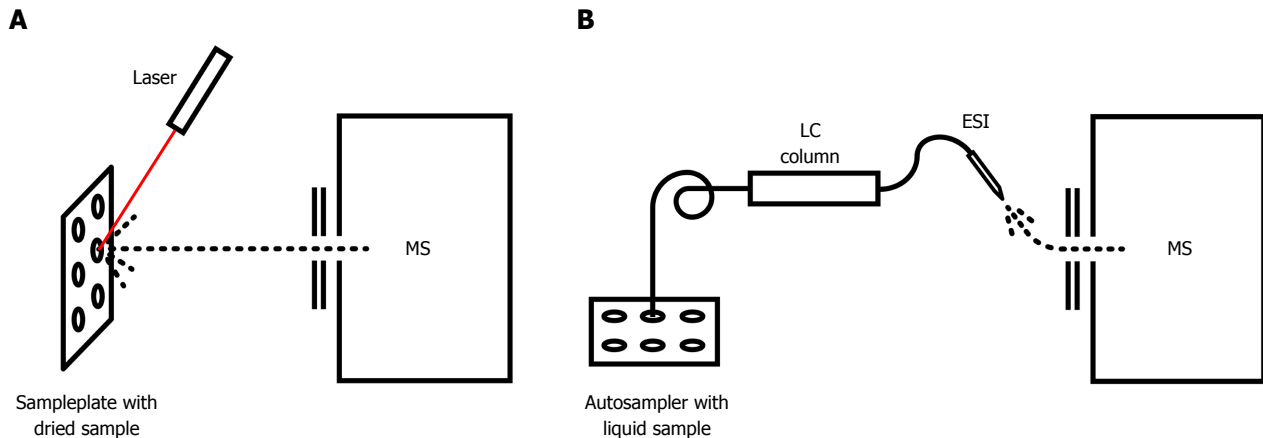


Figure 2 Two commonly used mass spectrometry techniques. A: Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MS), where the peptide or protein sample is dried on a target plate. Subsequently, a laser is used to evaporate the dried sample, and the generated gas phase ions are analyzed by the mass spectrometer; B: Liquid chromatography (LC)-electrospray ionization (ESI) MS, where the liquid peptide (or protein) sample is separated on a LC column, and sequentially eluted often over several hours. The eluted peptides are injected directly into the mass spectrometer by ESI and analyzed.

as the involvement of host-microbe interactions in the IBDs have been suggested^[54]. The cells were lysed, and the lysates were chemically modified using chemical labels to allow for a relative comparison between the protein abundances measured by MS. Using ESI LC-MS, the group recorded 10921 peptide fragments mass spectra, from which they were able to identify 2000 proteins. Two hundred and sixty four proteins had a known biological function and were found to have at least a 2-fold abundance difference between infected and non-infected, roughly half were more abundant post infection. Some of the MS-findings were verified with western blots, and significant changes were found in amount of actin-related proteins before and after infection.

Even though ESI LC-MS has advantages in terms of high-throughput, many biomarker studies have successfully employed MALDI-TOF MS protein identification in IBDs. In 2006, Hsieh *et al*^[68] applied discovery based proteomics using such a platform. The group analyzed the etiology and pathogenesis of UC using colonic biopsies to detect any significant difference in the protein profiles. The biopsies were obtained from four UC patients, three patients with nonspecific infectious colitis and five individuals with no obvious colonic disease. The proteins were separated by 2D-PAGE and a total of 1000 protein spots were compared visually between the diseased *vs* normal colon mucosa tissues. Forty protein-spots were found to be consistently different in intensity. Spots of interest were cut from the gel, tryptic digestion was performed and 19 proteins were identified using MALDI-TOF MS. Hereof, 13 identified proteins were less abundant in the UC-group and six proteins were more abundant. Eight of the less abundant proteins were identified as being mitochondrial proteins, suggesting that mitochondrial dysfunction might be involved in UC.

A year later in 2007, Shkoda *et al*^[69] also identified a potential association between dysfunction in the en-

ergy metabolism and IBDs. The group applied a similar strategy and platform to investigate the loss of intestinal cell function, a critical component in the initiation and perturbation of chronic intestinal inflammation, and was the first to compare inflamed and non-inflamed tissue from the same patient. Intestinal cells were purified from intestinal tissue obtained from patients suffering from CD, UC, and colon cancer. The proteins were separated by 2D-PAGE and analyzed by MALDI-TOF MS and western blotting. 41 proteins were found to be differently abundant between inflamed and non-inflamed tissue, including proteins associated with signal transduction, stress response and energy metabolism. Thirty-two percent of all detected differentially regulated proteins associated with IBD were involved in energy metabolism. In 2007, Meuwis *et al*^[70] published the first proteomic serum profiling study using SELDI-TOF MS in IBD, a variation of MALDI-TOF MS. The study included 30 patients with CD, 30 patients with UC, 30 inflammatory controls and 30 healthy controls. By characterizing the serum only by the *m/z* signals and not identified proteins with SELDI-TOF MS, the group was able to differentiate CD from UC with sensitivity of 85% (51/60) and specificity of 95% (57/60). Several of the unidentified signals were subsequently identified by MALDI-TOF MS, western blotting, and ELISA assay. Four biomarker candidates were identified: platelet aggregation factor 4 (PF4), myeloid related protein 8, fibrinopeptide A and haptoglobin alpha-2 subunit. All four proteins are known acute inflammatory markers to be expected in the IBDs, but the study succeeded in demonstrating that the separation of CD and UC patients based on serum markers is possible, highlighting the potential of serum profiling.

A year later, Meuwis *et al*^[70] used the same platform and strategy to analyze if serum from 20 CD patients could be used to predict response to infliximab treatment. Infliximab is a monoclonal antibody against TNF-alpha, and was the first anti-TNF-alpha agent accepted for

IBD treatment. The protein profiles were characterized in serum prior to and post treatment with SELDI-TOF MS. The group verified the four previous biomarkers, and especially increased amount of PF4 was associated with non-response to infliximab. However, the association could not be confirmed by ELISA, and did not correlate significantly with other disease markers. Even so, the study was able to predict responders with a sensitivity of 79% (55/70) and a specificity of 80% (56/70). Even though the study did not succeed in identifying a usable biomarker for the prediction of responders, the study highlighted the potential in proteomic studies and response marker discovery.

In 2007, Nanni *et al.*^[71] optimized the methodological approach used to evaluate serum with MALDI-TOF MS. Using a solid-phase bulk protein extraction protocol followed by MALDI-TOF MS, they analyzed serum from 15 CD, 26 UC and 22 healthy individuals and were able to separate the three groups with 97% prediction results. Two years later, Nanni *et al.*^[72] conducted a study using high-throughput ESI LC-MS to investigate protein variations in the intestinal epithelial cells from CD patients. However, in contrast to Hardwidge *et al.*^[67] in 2004 who used chemical labelling of the peptides to measure the relative abundances, Nanni *et al.*^[72] employed a label-free strategy, and relied on the accurate detection of the peptide masses. In this way, significant savings can be achieved for large studies and the sample preparation protocols simplified. Intestinal epithelial cells were isolated from samples originating from two CD patients and two control patients. The cells were lysed and the proteins were separated by 1D-PAGE, where the proteins are separated only in one dimension in contrast to 2D-PAGE, which allowed the entire visualized gel lane to be cut into pieces and digested with trypsin. The resulting peptides were analyzed by ESI LC-MS and by comparing the peptide intensities, relative protein abundances could be calculated. Proteins which were found to be more abundant in the epithelial cells from CD patients include heat shock protein 70, tryptase alpha-1 precursor as well as several proteins involved in inflammation processes. The nuclear protein Annexin A1, involved in the anti-inflammatory action, and the malate dehydrogenase enzyme was found to be less abundant. The feasibility of the biomarker candidates remains to be validated. However, of great importance is the demonstration of the utility of label-free ESI LC-MS analysis for the identification of differences in protein abundances for IBD.

In 2010, Hatsugai *et al.*^[73] performed the first study which successfully discriminated UC from CD completely. The group analyzed peripheral blood mononuclear cells from 17 UC patients, 13 CD patients and 17 healthy controls. The proteins were separated by 2D-PAGE and more than 1000 protein spots were detected in each gel. Five hundred and forty-seven protein spots were selected for the quantitative analysis, and 34 protein spots were significantly different between the UC and CD groups. Using 58 protein spots, the UC and CD patients could

be differentiated. The 58 protein spots were furthermore subjected to in-gel tryptic digestion followed by MALDI-TOF MS protein identification. Eleven of the proteins were successfully identified, and were found to be functionally related to inflammation, oxidation/reduction, the cytoskeleton, endocytotic trafficking and transcription. The profiles could, furthermore, predict disease severity and the UC patients' responses to treatment.

In 2011, M'Koma *et al.*^[74] analyzed mucosal and sub-mucosal layers of samples originating from Crohn's colitis (CC) and UC, using MALDI-TOF MS. Five unknown m/z MS signals were detected, which could separate the two groups. The study did not identify the origin of the signals, but highlighted the possibility of finding biomarkers in the intestinal tissue.

As mentioned earlier, even though we are far from having a complete picture of the intestinal micro-biome, changes in the bacterial composition have been detected in IBD. In 2012, Presley *et al.*^[75] investigated the host-microbe interaction at the intestinal mucosal-luminal interface of 14 CD patients, 21 UC, and 16 healthy controls. The mucosa prevents microorganisms from entering the host tissue. Using a novel saline-lavage technique, saline was injected during colonoscopy and extracted again to avoid interference from the intestinal layer contents resulting from a biopsy sample. The bacterial ribosomal RNA genes were analyzed by oligonucleotide fingerprinting and the proteins were analyzed by SELDI-TOF MS and MALDI-TOF MS. A combined proteome was constructed, constituting the proteomes from all detected organisms. Of the 3374 detected bacterial phylotypes, 35% significantly differentiated the diseases, indicating that host-microbe interactions might be involved in IBD, presenting new possibilities for diagnosis and therapy.

In 2013, Han *et al.*^[14] analyzed colonic tissue of Korean IBD patients in a high-throughput manner using ESI LC-MS and label-free quantitation. The study included four UC patients, three CD patients and two with inflammatory related polyps related to UC. The biopsies were homogenized and digested with trypsin without prior prefractionation and on average 324 proteins were identified for each group. Even though the number of identified proteins is relatively low considering the 2000 proteins Hardwidge *et al.*^[67] identified in 2004, 27 potential biomarkers were identified for UC, 37 biomarkers for CD and finally 11 proteins that were commonly associated with IBD. Three novel proteins, bone marrow proteoglycan, L-plastin and proteasome activator subunit 1 were identified as potential biomarkers for active CD. These biomarkers need validation, however, the feasibility of conducting high-throughput proteomics with label-free strategies in biomarker discovery was demonstrated.

A study published in 2013 by Seeley *et al.*^[76] investigated histological layers of 62 confirmed UC and CC tissues by MALDI-TOF MS. A total of 114 m/z MS signals were found to be statistically different between the two groups, however the signals have yet to be identified.

Finally, in 2013, Gazouli *et al.*^[77] published a study

where the response of 18 CD patients to infliximab treatment was correlated with known serum biomarkers. Serum samples were analyzed using 2D-PAGE, and 240 protein spot were selected for in-gel digestion and subsequent MALDI-TOF MS protein identification. The group was successful in identifying 15 proteins which were differentially present in the serum of CD patients depending on the response to infliximab. The proteins apolipoprotein A-I, apolipoprotein E, basic complement C4, plasminogen, serotransferrin, beta-2-glycoprotein 1, and clusterin were found to be more abundant in the patient groups with clinical and serological non-responders and responders, than in the group of patients with clinical and serological remission. Additionally, leucine-rich alpha-2-glycoprotein, vitamin D-binding protein, alpha-1B-glycoprotein and complement C1r subcomponent were found to be more abundant in the serum of the group of patients with remission. Interestingly, the group was unable to confirm the findings by Meuwis *et al.*^[70], that PF4 could be a biomarker for infliximab response, emphasizing that the biomarker candidates need further validation. Nonetheless, the study was successful in demonstrating the feasibility of identifying biomarkers in the serum usable to predict treatment outcome.

As apparent, many studies have successfully applied proteomic strategies to identify biomarkers, investigate IBD pathogenesis and identify prognostic markers in serum, stools, and tissue. Several biomarkers have been found (Table 2), most related to unspecific inflammation, and all biomarker candidates identified so far lacks follow-up validation studies. However, even though many of the identified biomarkers are related to inflammation, the studies have demonstrated the feasibility and potential of the proteomics platform in IBD, and given clues to the mechanisms of the IBDs. A few studies have successfully differentiated CD patients from UC patients. However, only based on unidentified m/z signals and not using identified protein or peptide biomarkers, from which the disease etiologies might be better explained. Nonetheless, these studies demonstrate the presence of usable biomarkers yet to be identified. Identified biomarkers hold the potential for designing diagnostic ELISA tests and protein array chips, where antibodies are used to detect the abundance of one or more antigens^[78,79]. Such arrays could constitute new clinical tools for diagnosis, prognosis and identify novel targets for therapy.

The studies have demonstrated the presence of biomarkers, in serum, in the intestinal tissue and in stools. Many studies have aimed at performing global discovery-based proteomics in the intestinal tissue, and it has been demonstrated that high-throughput techniques such as ESI LC-MS, employing labelling or label-free quantitation are feasible ways to identify biomarkers in highly complex samples. The advantage of high-throughput protein identification and quantification strategies are especially apparent when disease etiologies are to be examined.

Furthermore, few studies have investigated the possible association between various PTMs and the IBD dis-

ease etiologies. Such an association is known from other inflammatory diseases; an example being the inflammatory joint-disease rheumatoid arthritis (RA) where the PTM citrullination is known to be involved in the etiology^[80-83].

POSTTRANSLATIONAL MODIFICATIONS AS BIOMARKERS

Today, more than 200 distinct PTM's are known^[84]. The PTMs are, to a large extend, important for the physiological function of the protein and the half-life of PTMs range from milliseconds to years^[85]. Unfortunately, they are also often low abundance, highly diverse and complex, and thus can be challenging to detect and characterize^[25,27,86]. Hence, PTMs represent promising targets for biomarker discovery studies. For a review on protein regulation by PTMs in the IBDs, we refer to the work by Ehrentaut *et al.*^[5]. Common *in vivo* PTMs include phosphorylation, which is a reversible modification of the amino acids tyrosine, serine and threonine. Phosphorylation is known to be involved in activation and inactivation of enzyme activity, modulation of molecular interactions and cell signaling through specific domains. Acetylation can target any N-terminal, and it is believed that 84% of all human proteins undergo this modification^[87]. The PTM affects the protein stability, and histone acetylation is known to play a role in gene regulation. Glycosylation is another central PTM. It is reversible and known to be involved in cell-cell recognition and signaling, and regulation of proteins. Disulfide bond formation between two cysteines is a key element in the stabilization of proteins and protein complexes, such as, antibodies by forming intra- and intermolecular crosslinks. Deamidation of asparagine or glutamine is a possible regulator of protein-ligand and protein-protein interactions, and ubiquitination is a marker for protein recycling/destruction^[25]. Several PTMs are known to be involved in the inflammatory responses, and PTMs could be involved in the IBD disease etiologies. Lastly, citrullination is the irreversible deimination of arginine into citrulline, *in vivo* catalyzed by the peptidylarginine deiminases, a calcium binding family of enzymes^[88,89]. The exact role of the modification remains largely unknown, but the modification is believed to alter the fold of the proteins, change the protein polarity, and/or lead to denaturation in order to render the protein more prone to enzymatic degradation^[80,88,89]. Citrullination has been associated with several diseases, including Alzheimer's disease^[90], and RA where an anti-citrullinated protein antibody was identified^[80-83]. Smoking has been associated with increased citrullination, and smoking is the best known environmental factor for the development of RA^[91-95]. Several studies have, furthermore, associated smoking with an increased risk of developing CD and UC^[96-100]. In RA, it is believed that citrullination of proteins results in the generation of new antigens being presented to the immune system, which in turn triggers an autoimmune response^[83]. It therefore seems plausible that citrullination may have a similar role

in the IBDs as well as other inflammatory diseases. However, as with many PTMs the MS-driven detection of citrullinated proteins in a high-throughput manner is not straight forward^[84,101-104]. Nonetheless, if disease-specific citrullinated proteins could be identified, these could be utilized in ELISA or protein array chips for prognostics and/or diagnostics. An example of the utilization of a similar biomarker is the diagnosis of RA patients, where the presence of anti-citrullinated protein antibodies in the serum is used to detect the disease with a sensitivity of 71% and specificity of 95%^[80-83].

CONCLUSION

The diagnosis of UC and CD patients remains difficult, especially in the early stages of the diseases, and early and accurate diagnosis of IBD-patients is crucial. Several studies have successfully identified promising biomarkers in stools, serum and tissue, demonstrating the presence of IBD biomarkers. However, none of the identified biomarkers have been implemented in clinical daily use, and the diagnosis is based on a combination of disease history, colonoscopy inflammation biomarkers and histological evaluation.

Few studies have aimed at investigating the global proteome of intestinal tissue using high-throughput techniques such as ESI LC-MS, and the potential of such analysis seems immense. The recent development within the field of high-throughput protein identification using MS, now allows for identifying and quantifying several thousand proteins in a few hours of analysis time. Besides protein abundances, PTMs represent promising targets for biomarker discovery studies. An analysis of tissue, serum or stools therefore seems promising to identify novel biomarkers. Such information could be used to make accurate diagnostic and prognostic tools to differentiate patient groups and predict treatment responses. Antibodies against one or more identified diagnostic targets could be used in ELISA or protein array chips, which in turn can be used to detect the abundance of the given antigen. Besides aiding physicians in making a correct diagnosis and treatment strategy, knowledge of disease specific proteins and PTMs might identify disease pathways and new targets for therapeutic agents, leading to improved pharmaceutical drugs.

Conclusively, protein identification and quantification using mass spectrometry holds great promise for the identification of novel diagnostic and prognostic biomarkers for the IBDs, and might help explain the disease etiologies, ultimately leading to improved treatment strategies.

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WJG 20th Anniversary Special Issues (3): Inflammatory bowel disease

Inflammatory bowel disease of primary sclerosing cholangitis: A distinct entity?

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Abstract

This is a review of the characteristic findings of inflammatory bowel disease (IBD) associated with primary sclerosing cholangitis (PSC) and their usefulness in the diagnosis of sclerosing cholangitis. PSC is a chronic inflammatory disease characterized by idiopathic fibrous obstruction and is frequently associated with IBD. IBD-associated with PSC (PSC-IBD) shows an increased incidence of pancolitis, mild symptoms, and colorectal malignancy. Although an increased incidence of pancolitis is a characteristic finding, some cases are endoscopically diagnosed as right-sided ulcerative colitis. Pathological studies have revealed that inflammation occurs more frequently in the right colon than the left colon. The frequency of rectal sparing and backwash ileitis should be investigated in a future study based on the same definition. The cholangiographic findings

of immunoglobulin G4-related sclerosing cholangitis (IgG4-SC) are similar to those of PSC. The rare association between IBD and IgG4-SC and the unique characteristics of PSC-IBD are useful findings for distinguishing PSC from IgG4-SC.

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Key words: Primary sclerosing cholangitis; Primary sclerosing cholangitis; Inflammatory bowel disease; Inflammatory bowel disease-associated with primary sclerosing cholangitis; Immunoglobulin G4-related sclerosing cholangitis

Core tip: Inflammatory bowel disease (IBD)-associated with primary sclerosing cholangitis (PSC) (PSC-IBD) shows an increased incidence of pancolitis, mild symptoms, and colorectal malignancy. Although an increased incidence of pancolitis is a characteristic finding, some cases are endoscopically diagnosed as right-sided ulcerative colitis. Pathological studies have revealed that inflammation occurs more frequently in the right colon than the left colon. The cholangiographic findings of immunoglobulin G4-related sclerosing cholangitis (IgG4-SC) are similar to those of PSC. The rare association between IBD and IgG4-SC and the unique characteristics of PSC-IBD are useful findings for distinguishing PSC from IgG4-SC.

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INTRODUCTION

Primary sclerosing cholangitis (PSC) is a chronic inflammatory disease characterized by idiopathic fibrous obstruction^[1]. The fibrosis causes diffuse narrowing of the intrahepatic and extrahepatic bile ducts, and the resulting persistent biliary stasis leads to hepatic cirrhosis and a poor prognosis. Liver transplantation is indicated as a treatment for PSC. According to the diagnostic criteria for PSC proposed by the Mayo Clinic in 1999^[1] and 2003^[2], in addition to cholangiographic findings, the presence of inflammatory bowel disease (IBD) is important.

However, several related studies with contradictory results have recently been published^[3,4].

IgG4-related sclerosing cholangitis (IgG4-SC) has recently been established as a new clinical entity^[5]. The cholangiograms of IgG4-SC are occasionally similar to those of PSC^[6]. The differential diagnosis of PSC and IgG4-SC is important because IgG4-SC patients respond well to steroid therapy^[7].

In this study, we aimed to clarify the clinicopathological characteristics of PSC-IBD and the usefulness of PSC-IBD in the diagnosis of SC.

LITERATURE SEARCH

We conducted a literature search of English articles related to PSC-IBD, published between 2005 and March 2013, using the following keywords: “PSC,” “primary sclerosing cholangitis,” “PSC-IBD,” “IBD,” “immunoglobulin G4 (IgG4)-related sclerosing cholangitis (IgG4-SC),” “autoimmune pancreatitis,” and “IgG4-related disease (IgG4RD).” We connected the key words using “OR”. Pertinent articles obtained from the literature search were reviewed. All references were manually verified, and all reference lists in the retrieved articles were scrutinized to identify any additional articles that might have been missed in the PubMed search. As clinical data on PSC-IBD are limited, the authors also considered their own > 20-year clinical experience in the treatment of PSC-IBD. This study was primarily limited to adults patients, with the exception of the “frequency of PSC-IBD” section.

FREQUENCY OF PSC-IBD

PSC is strongly associated with IBD, and the prevalence of PSC-IBD is as high as 60%-80% in western countries^[8]. Approximately 80% of IBD is represented by ulcerative colitis (UC), 10% by Crohn's disease (CD), and 10% by indeterminate colitis^[9]. Conversely, only 2%-7.5% of IBD patients develop PSC^[8].

Previous reports from Europe and the United States have indicated that IBD complicates a high proportion of PSC cases. In Japan, IBD is found in only 21%-32% of PSC cases, according to surveys conducted by the

Japanese Society of Gastroenterology^[10] and the Japan Society of Hepatology^[11]. In a second nationwide analysis, only 125 of 388 patients (32%) had an established diagnosis of IBD, 79% of whom had UC, whereas only 6.4% were diagnosed with CD^[11]. Ang *et al.*^[12] also reported a low association rate of 20% (2/10) between PSC and IBD in Singapore. The association of PSC with IBD varies depending on geographical location, with higher rates in European and American populations, and a significantly lower association in Asian patients^[13]. However, the incidence of IBD in PSC patients in our series was higher (68.9%) than that already reported in Japan^[14]. We speculate that this high incidence was noted because only PSC patients who had undergone total colonoscopy at clinical onset were enrolled in our study. Yamagishi *et al.*^[15] also reported a higher incidence of IBD in PSC patients (93%) examined by colonoscopy. It is possible that we overlooked the endoscopic findings, as we did not perform a careful total colonoscopy because the symptoms of IBD are mild and the endoscopic findings of the colon show only slightly abnormal changes. The second national survey in Japan also reported that the incidence of IBD in PSC patients increased to 61% when only PSC patients examined by total colonoscopy were enrolled^[11]. Therefore, the frequency of PSC-IBD should be evaluated among patients undergoing careful total colonoscopy. This selection criterion might decrease the observed differences in frequency between eastern and western countries.

The age at clinical onset of IBD is controversial and has not been clarified. Loftus *et al.*^[9] reported that the mean age of IBD diagnosis was higher among PSC-IBD patients (32 years) compared with controls (28 years). In contrast, Brackmann *et al.*^[16] reported that IBD patients with PSC were significantly younger at the onset of IBD symptoms (PSC: 19 years *vs* no PSC: 29 years; $P = 0.04$), whereas the colitis-colorectal cancer interval was similar to that of IBD patients without PSC (17 years *vs* 20 years; $P = 0.236$). Joo *et al.*^[4] showed that PSC patients with UC presented with UC at a significantly earlier age (mean age: 24.5 years) compared with UC controls (mean age: 33.8 years).

Takikawa *et al.*^[10,11] discovered two peaks in the PSC age distribution, which has never been observed in other countries, and revealed that most Japanese PSC patients associated with IBD were adolescents or young adults. A recent study from Canada also reported two peaks in the PSC age distribution^[17]. Our previous study also showed a two-peaked age distribution, and that patients with PSC-IBD were significantly younger than PSC patients without IBD (33.6 years *vs* 58.9 years, $P < 0.001$)^[14].

Although the diagnosis of IBD precedes that of PSC in most patients, a recent study found that a shift in the timing of diagnosis of the two diseases has occurred in recent years, with PSC more often being diagnosed first. PSC was diagnosed before IBD in a recent

Table 1 character of inflammatory bowel disease-associated with primary sclerosing cholangitis

Ref./Nation	Year	Endoscopic/histological findings			Histological findings
		Extension of IBD	Backwash-ileitis	Rectal sparing	
Loftus <i>et al</i> ^[9] United States	2005	Total colitis 56/61 (92% vs CUC 54%)	19/37 (51% vs CUC 7%) by endoscopy	32/61 (52% vs CUC 6%) by endoscopy	
Joo <i>et al</i> ^[14] United States	2009	Total colitis 34/40 (85% vs CUC 45%)	10/24 (35.7% vs 26.9%) by histology	11/40 (27.5% vs 25%) by histology	
Sano <i>et al</i> ^[14] Japan	2010	Total colitis 6/20 (35% vs CUC 35%) Right-sided 11/20 (55% vs 3.3%) Left-sided 1/20 (5% vs 31.7%)	Not studied	Not studied	Significantly higher inflammation in the right colon
Jørgensen <i>et al</i> ^[24] Norway	2012	Total colitis 60/110 (55%) Right-sided 25/110 (23%) Left-sided 3/110 (3%)	11/93 (12%) by endoscopy 17/87 (20%) by histology	73/110 (66%) by endoscopy 70/107 (65%) by histology	Significantly higher inflammation in the right colon
Boonstra <i>et al</i> ^[3] The Netherlands	2012	Total colitis 207/380 (83%) Left-sided 9/380 (4%)	< 10%	< 10%	
Schaeffer <i>et al</i> ^[27] Canada	2013	Total colitis (IBD preceding PSC) Right-sided (PSC following IBD)			

CUC: Chronic ulcerative colitis; IBD: Inflammatory bowel disease; PSC: Primary sclerosing cholangitis.

cohort (2003-2007) when compared with a early cohort (1993-1997) (50% vs 35%, $P = 0.0009$)^[18].

MECHANISM OF PSC-IBD PATHOGENESIS

Several important observations, coupled with the strong association between certain human leukocyte antigen (HLA) haplotypes and the frequency of concurrent extrahepatic autoimmune disorders, support the concept that PSC is an immune-mediated phenomenon^[19,20]. Three UC susceptibility loci to be associated with PSC, harboring the putative candidate genes REL, IL2 and CARD9 were identified^[19]. A recent study reported 12 significant genome-wide associations outside the HLA region, 9 of which were new, increasing the number of known PSC risk loci to 16. Despite comorbidity with IBD in 72% of the included cases, 6 of the 12 loci showed significantly stronger associations with PSC than with IBD, suggesting overlapping yet distinct genetic mechanisms for these two diseases^[20].

The pathogenesis of PSC has been elucidated from the standpoint of PSC-IBD. Translocation of microbial flora across an inflamed, permeable gut with subsequent activation of the immune system and inflammation of the biliary tree is a hypothesized mechanism for the development of PSC. Activated intestinal lymphocytes enter the enterohepatic circulation and persist as memory cells that cause hepatic inflammation. Chemokines and adhesion molecules shared by the intestine and liver could contribute to immune cell binding at both sites^[21]. The observations that PSC can develop after colectomy^[22] and that IBD can develop after liver transplantation^[23] have led some investigators to suggest that aberrant homing of lymphocytes between the intestine and liver could be involved in the pathogenesis of PSC^[21]. Three recent studies indirectly supporting this theory have been published. Patients who received a liver trans-

plant had lower clinical and histological IBD activity than the non-transplant group^[24]. Marelli *et al*^[25] reported that progressive PSC requiring liver transplantation is associated with a milder course of UC, including reduced disease activity and less use of steroids, azathioprine, and surgery. Navaneethan *et al*^[26] reported that severe, progressive PSC requiring liver transplantation appeared to reduce the disease activity of UC and the need for colectomy.

However, these theories cannot explain why only 2%-7.5% of IBD patients develop PSC^[8], whereas PSC is strongly associated with IBD, or why CD is less associated with PSC. It is also unclear why immunosuppression does not improve PSC.

CLINICOPATHOLOGIC CHARACTERISTICS OF PSC-IBD

Previous studies have suggested that PSC-IBD differs from IBD without PSC in several aspects. Table 1 summarizes the reports concerning PSC-IBD^[3,4,9,14,24,27]. PSC-IBD has been reported to show an increased incidence of pancolitis, rectal sparing, backwash ileitis, mild symptoms, and colorectal malignancy^[8]. However, these results are controversial. All of the investigators agree that inflammation involved in PSC-IBD is milder than that in typical UC without PSC. Moreover, the majority of PSC-IBD patients show no or few IBD symptoms. Moayyeri *et al*^[28] reported that the number of hospitalizations and courses of steroid therapy decreased significantly in UC-PSC patients compared with UC controls. Our study also indicated that none of the enrolled patients had a severe clinical course, and that half of them were asymptomatic^[14].

However, varied findings concerning the extent of PSC-IBD have been reported. Although some authors have reported that pancolitis is characteristic of PSC-IBD^[3,4,9], others have insisted that right-sided IBD is

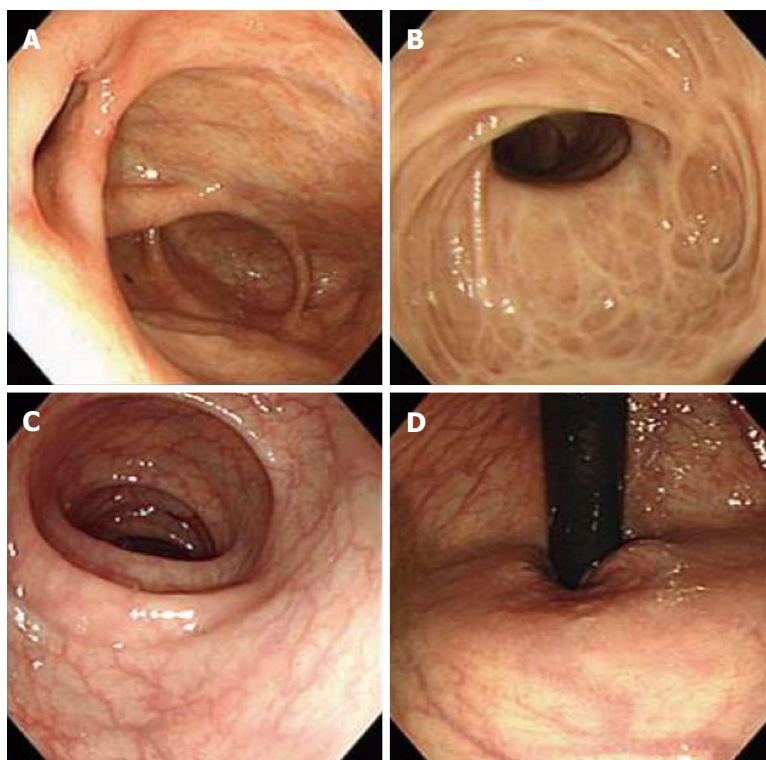


Figure 1 Colonoscopic findings at clinical onset. A: Cecum; B: Ascending colon; C: Sigmoid colon; D: Rectum. A 43-year-old female patient diagnosed with asymptomatic, concurrent primary sclerosing cholangitis-inflammatory bowel disease. The first colonoscopy showed multiple white scars in the ascending colon and right-sided transverse colon and no abnormal findings in the left-sided transverse colon, descending colon, sigmoid colon, or rectum.

characteristic^[14,24]. A recent report concluded that when IBD precedes PSC, pancolitis is common, whereas when PSC precedes IBD, right-sided IBD is common^[27].

Two reports revealed that the degree of inflammation was stronger in the right colon than the left colon using the histological studies. The first report demonstrated severe inflammatory cell infiltration in the cecum and ascending colon of PSC-IBD patients, whereas the degree was mild in the rectum/descending colon ($P = 0.0012$); goblet cell disappearance was also observed more frequently in the cecum/ascending colon than in the rectum/descending colon ($P < 0.05$)^[14]. The second report revealed that the histopathological signs of inflammation involved the right colon in 86% of patients and were purely right-sided in 23%. The frequency of inflammatory findings was higher in the right colon than the left colon ($P < 0.01$), but the general level of inflammatory activity was low^[24]. They also reported that inflammatory findings were more frequent on histology than on endoscopy.

These discrepancies may be due to differences in the geographical location between studies or the method used to assess total colitis based on the maximal extent of colitis at any time during the study period. Fluctuations in the extent of inflammation may therefore explain the high frequency of total colitis^[24,29].

The frequency of rectal sparing and backwash ileitis differs between reports, with the frequency of backwash

ileitis ranging from $< 10\%$ to 51% . Jørgensen *et al.*^[24] speculated that repeated examination or different patient selection criteria account for these differing results, and also reported that the frequency of rectal sparing on histological examination (20%) was higher than that on endoscopic examination (12%). Nakazawa *et al.*^[5] speculated that the different results were due to the definition and criteria of backwash ileitis.

The frequency of rectal sparing ranges from $< 10\%$ to 66% . Loftus *et al.*^[9] reported that rectal sparing was observed in 52% of UC-PSC patients compared with only 6% of UC patients without PSC on endoscopy. Jørgensen *et al.*^[24] reported that rectal sparing was observed in 66% of patients on endoscopy and was consistent with the results of pathological examination. In their study, the patients were considered to have rectal sparing if the inflammation involved the rectum but was less severe than that of the more proximal area of the colon. Joo *et al.*^[4] reported that rectal sparing was observed in only 27.5% of patients on histologic studies. Rectal sparing was defined as predilection if all biopsies from the rectum or the entire rectal mucosa from a resection specimen showed histologically normal mucosa. Rectal sparing was considered relative if at least one biopsy from the rectum at any time period or one portion of the rectal mucosa from the colonic resection specimen showed histologic features of chronicity, but at least mild activity was present in the areas of the mucosa proximal

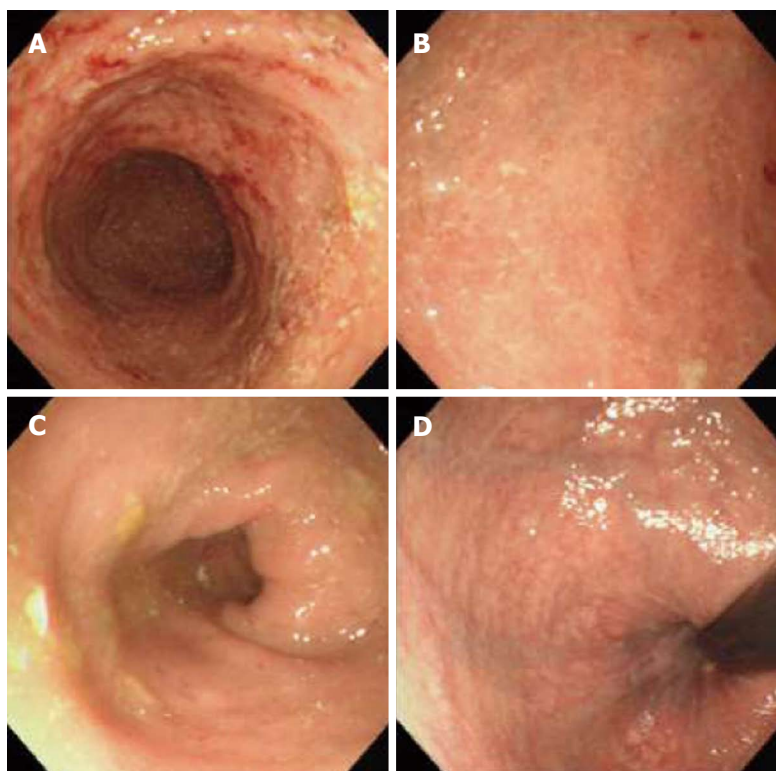


Figure 2 Colonoscopic findings seven months later. A: Cecum; B: Ascending colon; C: Sigmoid colon; D: Rectum. A repeat colonoscopy seven months later showing inflamed mucosa with multiple erosions and redness from the ascending colon to the right-sided transverse colon. Mucosal vessels are clearly visible in the descending colon, sigmoid colon, and rectum.

to the rectum.

The extent of colon involvement, rectal sparing, and backwash ileitis should be defined internationally to clarify the characteristics of PSC-IBD.

In our experiences with endoscopy, inflammation was more severe on the right, and rectal sparing appeared to be present on endoscopy (Figures 1 and 2), by contrast, less inflammatory cell infiltrations was observed in the rectum on histology. These findings are very useful in the diagnosis of PSC. In the presence of multiple biliary stenoses on magnetic resonance cholangiography, inflammatory findings dominant in the right colon and rectal sparing by total colonoscopy, invasive endoscopic retrograde cholangiography can be avoided in clinical practice.

COLORECTAL NEOPLASIA IN PSC-IBD PATIENTS

PSC-IBD patients are at particularly high risk for the development of colorectal cancer. Boonstra *et al*^[3] estimated that the colorectal cancer risk was increased 10-fold in PSC-IBD patients compared with UC controls. Claessen *et al*^[29] have reported that patients with PSC-IBD have a high long-term risk of developing colorectal cancer, and that this risk is approximately three-fold higher than that of cholangiocarcinoma. In patients with PSC-IBD, the 10-year and 20-year risks of colorectal cancer have

been reported to be 14% and 31%, respectively. Among 75 PSC-IBD patients, PSC was the only independent risk factor for the development of colorectal cancer, and the overall survival rate without liver transplantation was also reduced^[30].

PSC-IBD patients tend to be younger at colorectal cancer diagnosis^[31]. Colorectal cancer develops at a much younger age in these patients (39 years, range: 26-64 years) compared with IBD controls (59 years, range: 34-73) ($P = 0.019$)^[3].

Colorectal cancer in PSC-IBD patients predominantly develops in the right colon. The tumors were located proximally to the splenic flexure in 18 (67%) patients with PSC and in 52 (36%) patients without PSC ($P = 0.006$)^[29]. Thackeray *et al*^[32] also reported that colorectal cancer is most prevalent in the proximal colon (65%). When such patients are diagnosed with cancer, they tend to have more advanced tumors than IBD patients without concurrent PSC. In their study, patients with PSC had significantly more tumors with an American Joint Committee on Cancer tumor stage of 3A or higher when compared with patients with IBD alone (61.5% and 38.5%, $P = 0.003$). The reason for the preferential right-sided location of colorectal cancer in PSC patients remains unknown, although it has been speculated that the overall increased frequency of colorectal cancer in these patients could be due to a cytotoxic effect on the colonic mucosa caused by an altered bile acid composi-

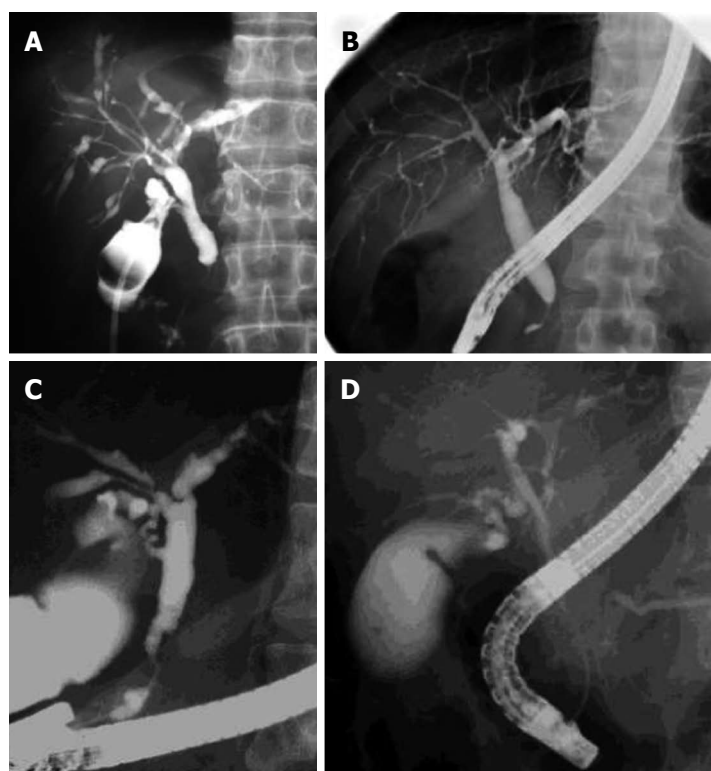


Figure 3 Cholangiographic examples of immunoglobulin G4-related sclerosing cholangitis and primary sclerosing cholangitis. Cholangiograms of immunoglobulin G4-related sclerosing cholangitis showing multiple stenoses in the intrahepatic ducts and stenosis in the intrapancreatic portion (A, B). Cholangiograms of primary sclerosing cholangitis showing a beaded appearance (C) and pruning of the intrahepatic ducts (C, D).

Table 2 Inflammatory bowel disease association in sclerosing cholangitis *n* (%)

Ref.	IgG4-SC	PSC	
Nishino <i>et al</i> ^[44]	0/24 (0)	15/24 (62.5)	$P < 0.0001$
Zen <i>et al</i> ^[45]	0/17 (0)	5/5 (100)	$P < 0.0001$
Ghazale <i>et al</i> ^[46]	3/53 (6)	70%	
Mendes <i>et al</i> ^[41]	NA	Elevated group (<i>n</i> = 12) 6/12 (50) Normal group (<i>n</i> = 115) 97/115 (90)	
Nakazawa <i>et al</i> ^[25]	0/62 (0)	21/31 (68)	$P < 0.0001$

IgG4-SC: Immunoglobulin G4-related sclerosing cholangitis; PSC: Primary sclerosing cholangitis.

tion. The predominance of right-sided inflammation is also a predisposing factor^[24]. The two reports showing that the histologic findings of inflammation were higher in the right compared to the left colon in PSC-IBD patients are consistent with the preferential right-sided location of colorectal cancer^[14,24].

Previous studies have shown conflicting results regarding the course of IBD after liver transplantation in patients with PSC. Recent studies have shown that the increased risk of neoplasia is maintained after liver transplant and proctocolectomy. Hanounch *et al*^[33] reported that patients with PSC-IBD after liver transplantation had a similar rate of colon neoplasia compared to those without liver transplantation (34% *vs* 30%, $P = 0.24$) during a mean follow-up period of 54.7 ± 47.7 mo. Jørgensen *et al*^[24] showed that macroscopic colonic

inflammation was more frequent after liver transplantation than before transplantation. The rate of relapse after transplantation was higher than that before transplantation, and the overall clinical IBD activity was also increased. Immunosuppression affects IBD activity after liver transplantation in patients with PSC.

Early cancer detection through enrollment in surveillance programs is the only strategy available to decrease cancer risk^[31]. More extensive colitis with a concurrent mild or even asymptomatic course, resulting in diagnostic delay and a lower colectomy rate, may contribute to an increased risk of colorectal cancer development^[9]. With the initial diagnosis of PSC in subjects with IBD, immediate and annual surveillance colonoscopy and biopsy analysis, of the entire colon, are recommended^[32,34].

The role of ursodeoxycholic acid (UDCA) as a chemopreventive agent is controversial. A meta-analysis showed no significant protective association between UDCA use and colorectal neoplasia. However, there was a significant chemopreventive effect on the risk of advanced colorectal neoplasia (OR = 0.35, 95%CI: 0.17-0.73), and low-dose UDCA use (8-15 mg/kg per day) was associated with a significantly reduced risk of colorectal neoplasia^[35]. There were no significant differences in cholangiocarcinoma incidence between the high-dose UDCA (17-23 mg/kg per day) and placebo groups^[36]. A recent study showed that patients treated with high-dose UDCA had a significantly higher risk of developing colorectal neoplasia during the study compared with those who received placebo (HR = 4.4, $P = 0.02$)^[37].

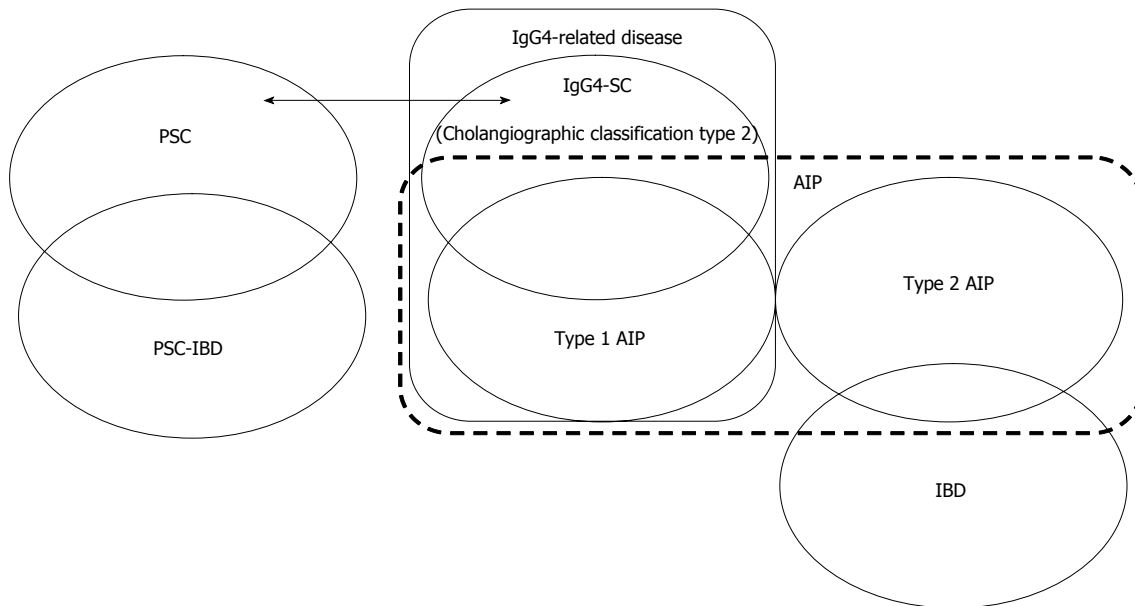


Figure 4 Correlation between inflammatory bowel disease and sclerosing cholangitis. PSC is frequently associated with characteristic PSC-IBD, whereas IgG4-SC is not associated with IBD. IgG4-SC is frequently associated with type 1 AIP, whereas type 2 AIP is frequently associated with IBD. PSC: Primary sclerosing cholangitis; PSC-IBD: IBD-associated with PSC; IBD: Inflammatory bowel disease; IgG4-SC: Immunoglobulin G4-related sclerosing cholangitis; AIP: Autoimmune pancreatitis.

THE USEFULNESS OF PSC-IBD CHARACTERISTICS IN THE DIAGNOSIS OF SCLEROSING CHOLANGITIS

Recently, IgG4-SC has attracted much attention following the emergence of clinical characteristics that distinguish it as a new clinical entity^[5]. IgG4-SC shows various cholangiographic features similar to those of pancreatic cancer, PSC, and cholangiocarcinoma^[7]. The characteristic cholangiographic features of IgG4-SC can be classified into four types based on the stricture location revealed by cholangiography and differential diagnosis^[6]. Type 2 IgG4-SC, in which stenosis is diffusely distributed throughout the intrahepatic and extrahepatic bile ducts, should be differentiated from PSC^[38] (Figure 3).

The differential diagnosis of PSC and IgG4-SC is important because patients with IgG4-SC show a good response to steroid therapy. We previously reported the differences between IgG4-SC and PSC^[7]. The age at clinical onset was significantly higher for IgG4-SC patients. Among the chief complaints in IgG4-SC, obstructive jaundice, reflecting marked concentric stenosis of the large bile duct, was most frequently observed. An elevated serum IgG4 level is a characteristic feature of IgG4-SC^[39]. An elevated serum IgG4 level and the association with type 1 autoimmune pancreatitis (AIP) are the most useful findings for discriminating between IgG4-SC and PSC^[6]. However, elevation of the serum IgG4 level alone is not useful because some PSC cases also show increased IgG4 levels. In addition, some IgG4-SC cases are not associated with AIP^[40]. Mendes *et al*^[41] measured the serum IgG4 levels in 127 patients with PSC and

found that it was elevated in 12 patients (9%). Björns-son *et al*^[42] reported elevated serum IgG4 levels in 12% of 285 patients with classic PSC. We performed a multicenter study in Japan to establish a cutoff value to differentiate IgG4-SC from controls^[43]. Serum IgG4 levels were compared between 56 patients with type 2 IgG4-SC and 110 patients with PSC. The serum IgG4 levels of the IgG4-SC patients were significantly higher than those of the PSC patients (799 ± 800 mg/dL *vs* 68.7 ± 86.0 mg/dL, respectively, $P < 0.001$). When we set the IgG4 cutoff value at 135 mg/dL, the sensitivity, specificity, and accuracy were 94.5%, 85.0%, and 90.5%, respectively. We also identified 13 of the 110 PSC patients (11.8%) with IgG4 values higher than the cutoff value of 135 mg/dL^[43]. Further studies are expected to clarify the differences between IgG4-SC and PSC patients with high serum IgG4 levels.

In contrast, the diagnosis of PSC is difficult because there are no useful markers of PSC. As such, an association with IBD is a very useful finding in the diagnosis of PSC. The frequencies of IBD associated with IgG4-SC are summarized in Table 2^[5,41,44-46]. No association between IBD and IgG4-SC has been reported, which is in contrast to the strong association between IBD and PSC (60%-80%)^[5,44-46]. One study reported that 6% of IgG4-SC cases were associated with IBD^[46], as IBD is common in western countries. We are unable to describe the details of IBD-associated IgG4-SC, although we speculate that the unique characteristics of PSC-IBD can be used to distinguish PSC from IgG4-SC.

IgG4-SC is frequently associated with type 1 AIP^[5]. Type 1 AIP is a different clinical entity from type 2 AIP, which is closely associated with IBD. Type 2 AIP is not

associated with IgG4-related diseases, including IgG4-SC, and elevated serum IgG4 levels are not observed^[47]. Clinically diagnosed IBD is incorporated in the diagnostic criteria for type 2 AIP in international consensus diagnostic criteria for autoimmune pancreatitis^[47]. These complicated associations are illustrated in Figure 4. There are no reports concerning the characteristics of IBD associated with type 2 AIP. If IBD associated with type 2 AIP shows characteristic findings, they might be useful not only in discriminating type 2 AIP from type 1 AIP, but also in elucidating the mechanism of sclerosing cholangitis.

In summary, PSC-IBD is associated with an increased incidence of pancolitis, mild symptoms and colorectal malignancy. Although an increased incidence of pancolitis is also a characteristic finding, some cases are endoscopically diagnosed as right-sided UC. Pathological studies have revealed that the findings of inflammation were more prevalent in the right colon than the left colon. The frequency of rectal sparing and backwash ileitis should be investigated in a future study based on the same definition. The rare association between IBD and IgG4-SC and the unique characteristics of PSC-IBD are useful for discriminating between PSC and IgG4-SC. In particular, when IBD is characterized by right-sided UC, rectal sparing, and backwash ileitis, the possible diagnosis of PSC-IBD should be considered. In the presence of multiple biliary stenoses on magnetic resonance cholangiography, inflammatory findings dominant in the right colon and rectal sparing by total colonoscopy, invasive endoscopic retrograde cholangiography can be avoided in clinical practice.

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WJG 20th Anniversary Special Issues (3): Inflammatory bowel disease

Dysregulation of mucosal immune response in pathogenesis of inflammatory bowel disease

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Abstract

Inflammatory bowel disease (IBD) includes Crohn's disease and ulcerative colitis. The exact etiology and pathology of IBD remain unknown. Available evidence suggests that an abnormal immune response against the microorganisms in the intestine is responsible for the disease in genetically susceptible individuals. Dysregulation of immune response in the intestine plays a critical role in the pathogenesis of IBD, involving a wide range of molecules including cytokines. On the other hand, besides T helper (Th) 1 and Th2 cell immune responses, other subsets of T cells, namely Th17 and regulatory T cells, are likely associated with disease progression. Studying the interactions between various constituents of the innate and adaptive immune systems will certainly open new horizons of the knowledge about the immunologic mechanisms in IBD.

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Key words: Crohn's disease; Inflammatory bowel disease; Proinflammatory cytokines; T helper cells; T helper 17 cells; Ulcerative colitis

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Core tip: The etiology and pathology of inflammatory bowel disease (IBD) remain elusive, and dysregulation of the mucosal immune response toward commensal bacterial flora together with genetic and environmental factors may play important roles in the pathogenesis of IBD. A better understanding of the mechanisms of immune responses in the intestinal mucosa will provide new insights into the pathogenesis of IBD, and shed some light on targeted immune therapy for this disease.

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INTRODUCTION

The etiology and pathogenesis of inflammatory bowel disease (IBD) remain elusive, and accumulating evidence has indicated that sustained intestinal infections, mucosal barrier defects, mucosal immune dysregulation, genetic and environmental factors are involved in the disease process^[1-4]. Among these, the dysfunction of the mucosal immune system plays an important role in the pathogenesis of IBD (Figure 1). Among a variety of inflammatory cells in the gut, mucosal CD4⁺ T cells are thought to play a central role in both the induction and persistence of chronic inflammation by producing proinflammatory cytokines. Previous studies have indicated that T helper (Th) 1-related cytokines [e.g., tumor necrosis factor (TNF), interferon (IFN)- γ , interleukin (IL)-12] as well as Th17-associated cytokines (e.g., IL-17A, IL-21, IL-23) are markedly increased in inflamed mucosa of Crohn's disease

(CD) patients, whereas the cytokine profiles in inflamed areas of ulcerative colitis (UC) patients seem to exhibit increased production of the Th2-associated cytokines such as IL-4 and IL-13^[1-3]. These proinflammatory cytokines are potent *in vitro* stimulators of intestinal mucosal effect or functions, including T cell and macrophage proliferation, adhesion molecule expression, chemokine expression, and secretion of other proinflammatory cytokines.

ABNORMAL IMMUNE RESPONSE IN THE INFLAMED MUCOSA OF IBD PATIENTS

Antigen-specific activation of various lymphocytes within the intestinal mucosa by enteric pathogens is an important feature of IBD immunopathology^[1-4]. Under physiological conditions, a large number of innate and immune cells are located in the intestinal lamina propria, such as T, B, natural killer (NK), NKT cells, macrophages (Mφ), dendritic cells (DCs), mast cells, neutrophils, eosinophils, as well as stromal cells (such as fibroblasts). It is actually surprising that the large lymphoid system in the intestine coexists so peacefully with the external environment, a single epithelial layer away from the luminal microbial flora. However, under inflammatory conditions, a large number of activated immune cells infiltrate into the intestinal mucosa. These immune cells and some stromal cells not only express high levels of adhesion molecules and auxiliary signal molecules (such as CD54, CD62L), but also express high levels of inflammatory mediators and chemokine receptors (such as CCR5, CCR6, and CCR9) and integrin (such as integrin $\alpha 4\beta 7$). Moreover, fibroblasts and capillary endothelial cells in the intestinal mucosa also express high levels of chemokines, selectins (*e.g.*, selectin E) and intracellular adhesion molecule-1 (ICAM-1, or CD54), which further induce intermolecular interactions of leukocytes in the blood circulation to migrate into the intestinal mucosa, and promote local inflammatory response^[1-4].

Evidence has demonstrated that CD4⁺ T cells isolated from inflamed mucosa of CD patients, when stimulated *in vitro*, are able to produce large amounts of Th1/Th17-associated proinflammatory cytokines (*e.g.*, IFN- γ , TNF, and IL-17A), while in UC inflamed tissue CD4⁺ T cells and NK T cells secrete high levels of Th2-related cytokines (*e.g.*, IL-4 and IL-13) and Th17-associated proinflammatory cytokines (*e.g.*, IL-17A)^[1-4]. The unbalance of pro/antiinflammatory cytokines contributes to intestinal mucosal inflammation. Recent studies have found that some proinflammatory cytokines (*e.g.*, IL-12, IL-18, IL-21, and IL-23) are significantly increased in inflamed mucosa of CD patients^[5-7], and that some inhibitory cytokines (*e.g.*, TGF- β , IL-10, IL-25, IL-33, and IL-37) are significantly reduced. Moreover, loss of forkhead proteins (Foxp)3⁺ regulatory T cells (Treg) and FoxP3 IL-10⁺ CD4⁺ cells are also found in the inflamed mucosa of IBD patients, and these events result in not maintaining intestinal mucosal immune tolerance and further promoting local intestinal mucosal immune response, leading to

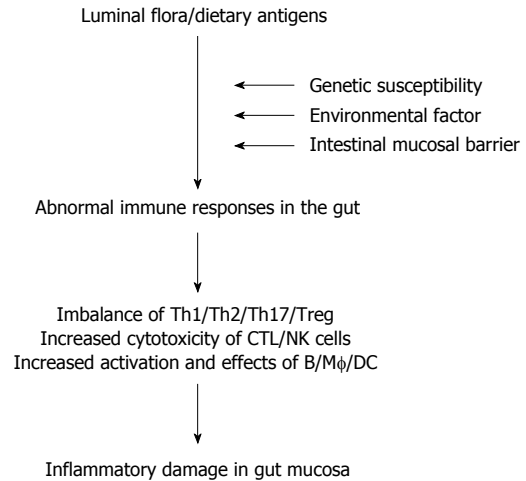


Figure 1 Pathogenesis of inflammatory bowel disease. DC: Dendritic cell; NK: Natural killer; Th: T helper; Treg: T regulator cell; CTL: Cytotoxic T lymphocyte; Mφ: Macrophages.

the intestinal mucosal injury^[8].

PLEIOTROPIC ROLE OF IL-21 IN IMMUNE RESPONSE

IL-21 is a member of the IL-2 family of cytokines, expressed mainly by CD4⁺ T cells, including Th1, Th2, and Th17 cells (Figure 2)^[9,10]. IL-21 receptor (IL-21R) is structurally related to IL-2R and IL-15R, and is expressed in T, NK, B cells, and DCs^[9]. IL-21 exhibits a pleiotropic capacity to regulate T cell differentiation and function, enhances clonal expansion of antigen-activated naive CD4⁺ and CD8⁺ T cells, and induces the expression of genes encoding IL-12R, IL-18R, IFN- γ , IL-2R α , and the Th1-associated transcription factor T-bet in activated memory T cells^[11,12]. IL-21 is also associated with the Th2-mediated immune response and plays a role in inhibiting the differentiation of naive Th cells into IFN- γ -producing Th1 cells. In synergy with IL-15, Fc γ 3 ligand, and stem cell factor, IL-21 promotes human NK cell maturation and activation. It exerts further biological functions in B cells, regulates differentiation and antibody production, including production of all IgG isotypes, and synergizes with anti-CD40 mAb to stimulate B-cell activation, clonal expansion, and maturation (Figure 2)^[13-15].

In recent years, IL-21 has been found to be produced in excess in the intestine of IBD patients and may be involved in the pathogenesis of human IBD^[16-18]. When mucosal T cells from CD patients are activated *in vitro* with anti-CD3 in the presence of either a neutralizing anti-IL-21 antibody or an IL-21R-IgG fusion protein, the production of both IL-17A and IFN- γ is reduced. These results together with the demonstration that IL-21-deficient mice are resistant against Th1/Th17 cell-driven colitis support the key role of IL-21 in positively regulating Th1 and Th17 cell-associated inflammatory pathways. IL-21 exerts further biological functions that could contribute to its proinflammatory effect in the gut. For

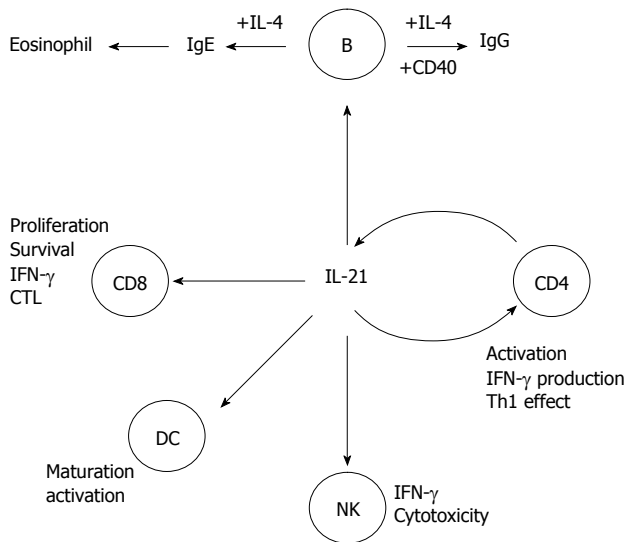


Figure 2 Pleiotropic role of interleukin-21 in immune responses. IFN: Interferon; IL: Interleukin; CD: Crohn's disease; DC: Dendritic cell; NK: Natural killer; CTL: Cytotoxic T lymphocyte.

example, IL-21 stimulates stromal cells to produce tissue-degrading proteases and enhances secretion of the T-cell chemoattractant macrophage inflammatory protein-3α by intestinal epithelial cells. IL-21 potentiates the expression of Th1-related transcription factors and IFN-γ in T and NK cells and the cytotoxic activity of NK cells. IL-21 also inhibits the peripheral differentiation of Tregs and makes CD4⁺ T cells resistant to Treg-mediated immune suppression. Therefore, through the multiple pathways IL-21 can damage the gut, and neutralizing IL-21 may have a therapeutic potential in the management of IBD^[13,18].

We have also investigated expression of IL-21R in inflamed mucosa of IBD patients and evaluated its role in the induction of NK cell cytotoxicity and activation as well as Th17 differentiation^[5]. The results have shown that IL-21R-positive cells are significantly increased in inflamed mucosa of IBD patients compared with healthy controls, and IL-21R is mainly expressed in freshly isolated peripheral blood (PB)- and lamina propria (LP)-CD4⁺, CD8⁺ T, B, and NK cells. When stimulated with immobilized human IgG and IL-21, PB-NK cells from IBD patients produce higher levels of IFN-γ and TNF than those from controls. IL-21-primed IBD NK cells show a more potent antitumor cytotoxicity to NK-sensitive K562 cells than controls. Moreover, PB-T and LP-T cells from IBD patients produce larger amounts of proinflammatory cytokines (e.g., TNF and IFN-γ) than those from controls when stimulated with IL-21 and anti-CD3. Importantly, IL-21 facilitates IBD CD4⁺ T cells to differentiate into Th17 cells^[5]. In our further study, we have also evaluated the role of anti-TNF mAb (infliximab, IFX) in regulating IL-21 expression and Th17 cell infiltration in the intestinal mucosa of CD patients. Twenty-six CD patients were treated with IFX at weeks 0, 2 and 6. IL-21 and Th17 cells were found to be expressed highly

in inflamed mucosa of active CD patients compared with healthy controls. Ten weeks after IFX infusion, CD activity index, erythrocyte sedimentation rate, serum C-reactive protein (CRP) and intestinal mucosal healing were improved markedly in CD patients. Moreover, IL-21 expression and Th17 cell infiltration were also found to be significantly decreased compared with those before IFX therapy^[19]. These data indicate that IL-21 plays an important role in the pathogenesis of IBD.

PROINFLAMMATORY ROLE OF IL-23 IN THE PATHOGENESIS OF IBD

Recent advances have also indicated that IL-23, mainly produced by macrophages, is one of the critical cytokines in IBD and is essential for promoting chronic intestinal inflammation^[20,21]. IL-23 and IL-12 are members of a small family of proinflammatory heterodimer cytokines, sharing a common p40 subunit covalently linked to a p35 subunit to form IL-12 or to a p19 subunit to form IL-23. IL-12R is comprised of an IL-12Rβ1 and IL-12Rβ2 subunit, whereas the receptor for IL-23 consists of the IL-12Rβ1 subunit and a novel component termed IL-23R^[20], which is expressed predominantly on T, NK, and NKT cells and to a smaller extent, on monocytes, macrophages, and DCs. After binding to the IL-23R, IL-23 preferentially induces memory T cell activation. IL-23 exhibits some similar biological activities to IL-12, however, in comparison with IL-12 with profound induction of the Th1 immune response, as well as promotion of cytotoxic, antimicrobial, and anti-tumor responses, IL-23 is found to play a critical role in the maintenance of immune response by controlling T cell memory function and by influencing the proliferation and survival of IL-17-producing Th17 cells^[22,23]. Moreover, recent work has also shown that IL-23 could induce naive CD4⁺ T cells to secrete IL-22, indicating that IL-23 is also associated with the differentiation of naive CD4⁺ T cells^[24].

Previous studies with murine colitis models have demonstrated a requirement for IL-23 in the development of intestinal mucosal inflammation. IL-23p19 subunit knockout results in a decrease of proinflammatory cytokines (e.g., TNF, IFN-γ, IL-6, and IL-17) and the presence of less intestinal mucosal inflammation^[25,26]. Moreover, *in vivo* blockade of IL-23 using anti-IL-23p19 mAb or its inhibitor STA-5326 could inhibit chronic intestinal inflammation in a colitis model and down-regulate a Th1-mediated immune response^[26,27]. Elevation of IL-23p19 transcript levels has been observed in inflamed mucosa of IBD patients, and its expression is correlated with the severity of endoscopic lesions^[28]. Recent work has demonstrated that myeloid DCs from mesenteric lymphoid nodes of CD patients, when stimulated with exogenous microbial antigens *in vitro*, produce higher levels of IL-23p19 than UC patients and healthy controls^[29].

In order to investigate the pathogenic role of IL-23 in the induction of mucosal inflammation in IBD, we have analyzed IL-23p19 expression in inflamed mucosa

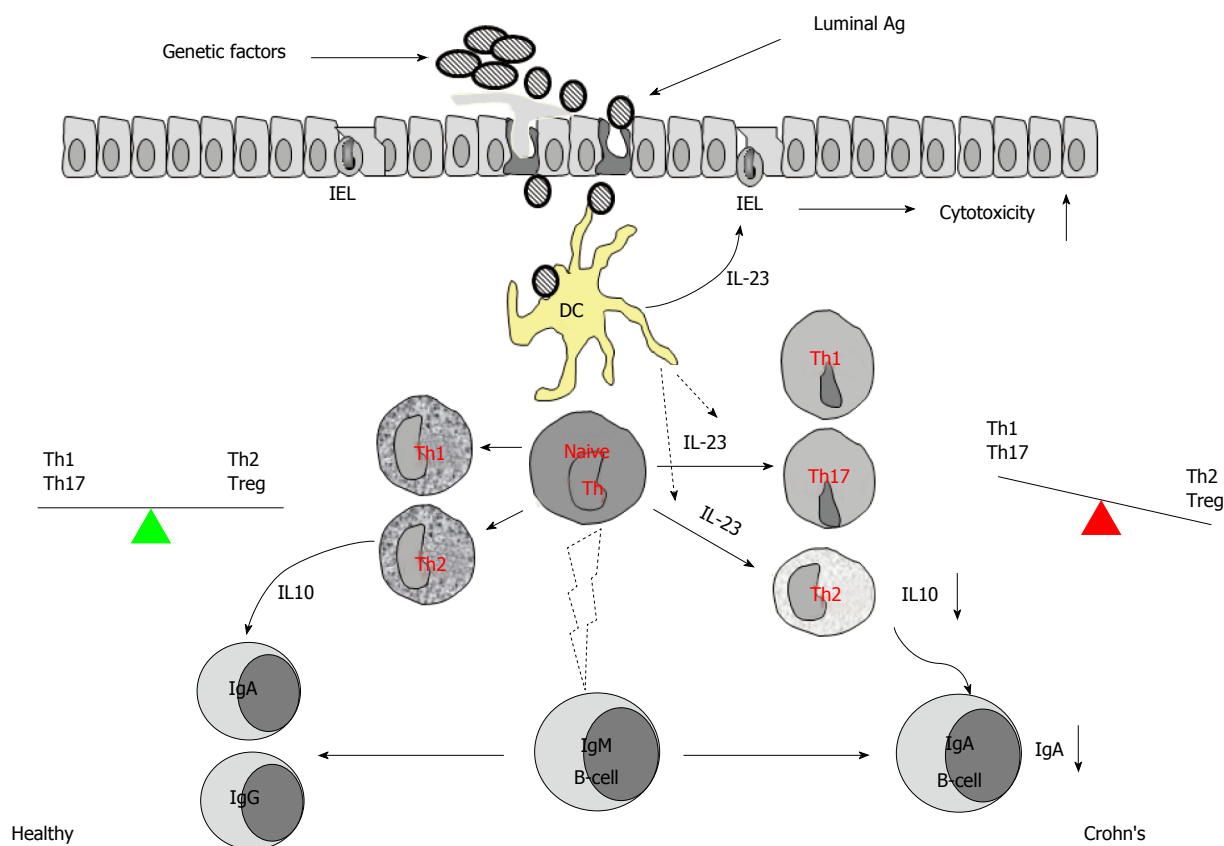


Figure 3 Overexpression of interleukin-23 in inflamed mucosa of patients with inflammatory bowel disease weakens the intestinal defensive barrier and disturbs the immune regulation in intestinal mucosa. IEL: Intestinal epithelial lymphocyte; IL: Interleukin; Th: T helper; Treg: T regulator cell; DC: Dendritic cell.

of IBD patients and its role in the induction of intestinal epithelial lymphocyte (IEL) and NK cell activation as well as Th17 cell differentiation. Expression of IL-23p19 has been observed to be increased significantly in inflamed mucosa of CD patients compared with that in UC patients and healthy controls. IL-23R cells are increased significantly in PB- and LP-CD4⁺ and -CD8⁺ T and NK cells. IL-23 could markedly promote IBD IEL and NK cell activation and cytotoxicity and triggered IBD PB- and LP-T cells to secrete significantly higher levels of IFN- γ , TNF, IL-2, and IL-17A compared with healthy controls. IL-23 promotes IBD PB- or LP-CD4⁺ T cells to differentiate into Th17 cells. These data indicate that IL-23 plays an important role in the induction of IEL, NK, and T cell activation, proinflammatory cytokine secretion, and Th17 cell differentiation^[6]. In two IBD models there is excessive accumulation of short-lived neutrophils and inflammatory monocytes in the intestine. IL-23-driven colitogenic T cell program has been found to regulate upstream hematopoietic stem and progenitor cells (HSPC)^[30]. Targeted therapy directed against IL-23 may have a therapeutic role in treatment of IBD.

Additionally, we have also elucidated the further role of IL-23 in the suppression of IL-10 in the IBD intestinal mucosa^[7]. IL-10 is an important cytokine in the induction of Th2 response that plays a crucial role in adaptive immunity *via* the induction of specific antibody-

ies to eliminate the reinvasion of microbes and the absorption of microbial products. IL-10 is also one of the most effective immune regulatory cytokines contributing to maintaining the homeostasis of the body^[31,32]. Previous studies indicate that the production of IL-10 in the intestine of IBD patients is suppressed, but the underlying mechanism has not fully understood yet. Therefore, we examined the expression of IL-10, IL-23, and IgA in the surgically removed colon specimens and found that the levels of IgA and IL-10 were significantly lower, and both negatively correlated with IL-23 expression and the infiltration of inflammatory cells in the IBD mucosa. The production of IL-10 by lamina propria mononuclear cells was lower in the IBD group than the control, and these levels could be enhanced by blocking IL-23. The gene transcription of IL-10 was significantly suppressed in CD4⁺ T cells of IBD mucosa, and this phenomenon could be replicated *in vitro* by adding IL-23 in the culture of polarized Th2 cells^[7]. Thus we conclude that overexpression of IL-23 in the intestinal mucosa weakens the defensive barrier in the gut and disturbs the local immune regulation (IL-10 and Treg) (Figure 3).

POTENTIAL ROLE OF IL-25 IN THE DEVELOPMENT OF IBD

IL-25 (also known as IL-17E) is a distinct member of the

IL-17 family of cytokines, including IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (IL-25), and IL-17F. IL-25 shares the receptor IL-17 receptor homolog 1 (IL-17Rh1) (also named the IL-17RB) with IL-17B, although it binds with a much higher affinity. IL-25R is a 56-kDa single transmembrane protein and is expressed in Th2 central memory cells, eosinophils, monocytes, airways smooth muscle cells, fibroblasts, and endothelial cells. Evidence has shown that IL-25 is also involved in the immune responses in gut mucosa^[33]. Previous work has demonstrated that IL-25 is constitutively expressed by intestinal mucosal T cells of mouse strains (*e.g.*, BALB/c, C57BL/6 mice) that are resistant to helminth *Trichuris muris* infection, whereas IL-25-deficient mice on a genetically resistant background fail to develop a Th2-mediated immune response or eradicate *Trichuris* infection but develop severe infection-induced intestinal inflammation. Moreover, the immunopathology in *Trichuris*-induced IL-25-deficient mice is also associated with increased expression of IFN- γ and IL-17A in the mesenteric lymph nodes and cecum^[33]. Administration of IL-25 could prevent intestinal mucosal inflammation in experimental colitis induced with peptidoglycan, 2,4,6-trinitrobenzenesulphonic acid, or oxazolone in mice. These data indicate that IL-25, which promotes the differentiation and activation of Th2 cells in gut mucosa, plays a critical role in the attenuation of destructive intestinal inflammation^[33,34].

IL-25 has been also found to be decreased in the inflamed mucosa of IBD patients and could decrease the synthesis of IL-12 and IL-23 in the CD14⁺M ϕ from the inflamed mucosa of patients with CD *in vitro*^[35]. Therefore, IL-25 may be a negative regulator of inflammatory responses in the intestinal mucosa. However, the exact role of IL-25 in the development of IBD remains to be elucidated. Recently, we have also studied the role of IL-25 in the pathogenesis of IBD^[36]. The results have demonstrated that IL-25 is significantly decreased in the sera and inflamed mucosa of patients with active IBD compared with controls. The levels of IL-25 in inflamed mucosa and sera are inversely correlated with endoscopic disease activities and CRP, respectively, in IBD. IL-25 could markedly inhibit IBD CD4⁺ T cells to produce TNF, IFN- γ , and IL-17A but promote IL-10 secretion. IL-25 could suppress the differentiation of IBD CD4⁺ T cells into Th1 and Th17 cells but did not interfere with Th2 cell differentiation. Importantly, blockade of IL-10 secretion by IBD CD4⁺ T cells markedly attenuates the inhibitory role of IL-25 in modulating both Th1 and Th17 immune responses (Figure 4). Our study provides evidence that IL-25 is a critical anti-inflammatory cytokine in the pathogenesis of IBD and may be considered as a potential therapeutic agent for human IBD^[36].

POTENTIAL ROLE OF THE TH17/IL-17 AXIS IN THE PATHOGENESIS OF IBD

IL-17 has pleiotropic activities, functions through the adaptive and innate immune system to promote im-

mune response, and plays an important role in immune responses. The identification of the IL-17 family of cytokines as well as the IL-23-mediated expansion of IL-17-producing T cells has uncovered a new subset of Th cells, designated as Th17 cells. Th17 cells originate from naive CD4⁺ T cells in the presence of TGF- β and IL-6. Th17 cell differentiation does not require IL-17. The amplification and stabilization of Th17 are provided by IL-21 and IL-23. At the same time, the ROR γ t is identified as the master key regulator and transcription factor of Th17 cell differentiation^[37,38]. The IL-17 cytokine family includes six members, IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (or IL-25) and IL-17F, and act *in vitro* and *in vivo* as potent proinflammatory cytokines^[38]. IL-17A can induce the expression of proinflammatory cytokines (such as IL-6 and TNF), chemokines (such as KC, MCP-1 and MIP-2) and matrix metalloproteases, which mediate tissue infiltration and tissue destruction. It is also involved in the proliferation, maturation and chemotaxis of neutrophils^[38].

Evidence has shown that high numbers of Th17 cells are present in the colonic LP of the ileum and colon in conventionally raised mice, and that these cells are highly infiltrated in inflamed areas of colitic mice^[39,40]. Further analysis confirms that commensal gut flora contributes to the expansion of these CD4⁺ Th17 cells, leading to intestinal mucosal inflammation. In terms of mucosal immunity, the IL-23/IL-17 axis has been observed to play an important role in normal intestinal homeostasis.

To date, IL-17 and other Th17-associated cytokines (*e.g.*, IL-22 and IL-23) have been found to have protective or pathogenic effects dependent on other effective factors in local tissue. Recent work^[41] has also demonstrated that most of the transcripts for Th17-related cytokines are increased in IBD patients compared to normal controls, but more abundant in UC than in CD. In contrast, upregulation of IFN- γ mRNA is marked in CD LP CD4⁺ T cells. Up-regulation of IL-23p19 mRNA is detected in colonic mucosa from both UC and CD patients. The significance of Th17 immunity in UC is further supported by the finding that recombinant IL-23 actually enhances IL-17A production by LP CD4⁺ T cells in UC, but has a lesser effect on LP CD4⁺ T cells in CD^[42]. IL-17A is protective against dextran sodium sulfate (DSS)-induced colitis and colitis in the T cell transfer model, in which T cells are injected into lymphopenic mice. However, mice deficient in IL-17F are resistant to DSS-induced colitis. These data suggest that IL-17F, not IL-17A, is pathogenic in the gut. IL-17 may synergize with other inflammatory mediators in the gut. Recent studies have highlighted further potential heterogeneity within Th17 cell populations by demonstrating that some may even secrete IL-10^[43], a factor known to inhibit intestinal inflammation. Thus, it is possible that the actions of Th17 cells may differ dependently on other factors that may be present in the local environment. In the normal intestine, the primary function of Th17 cells may be like sentinels which contribute to maintaining epithelial barrier function, whereas

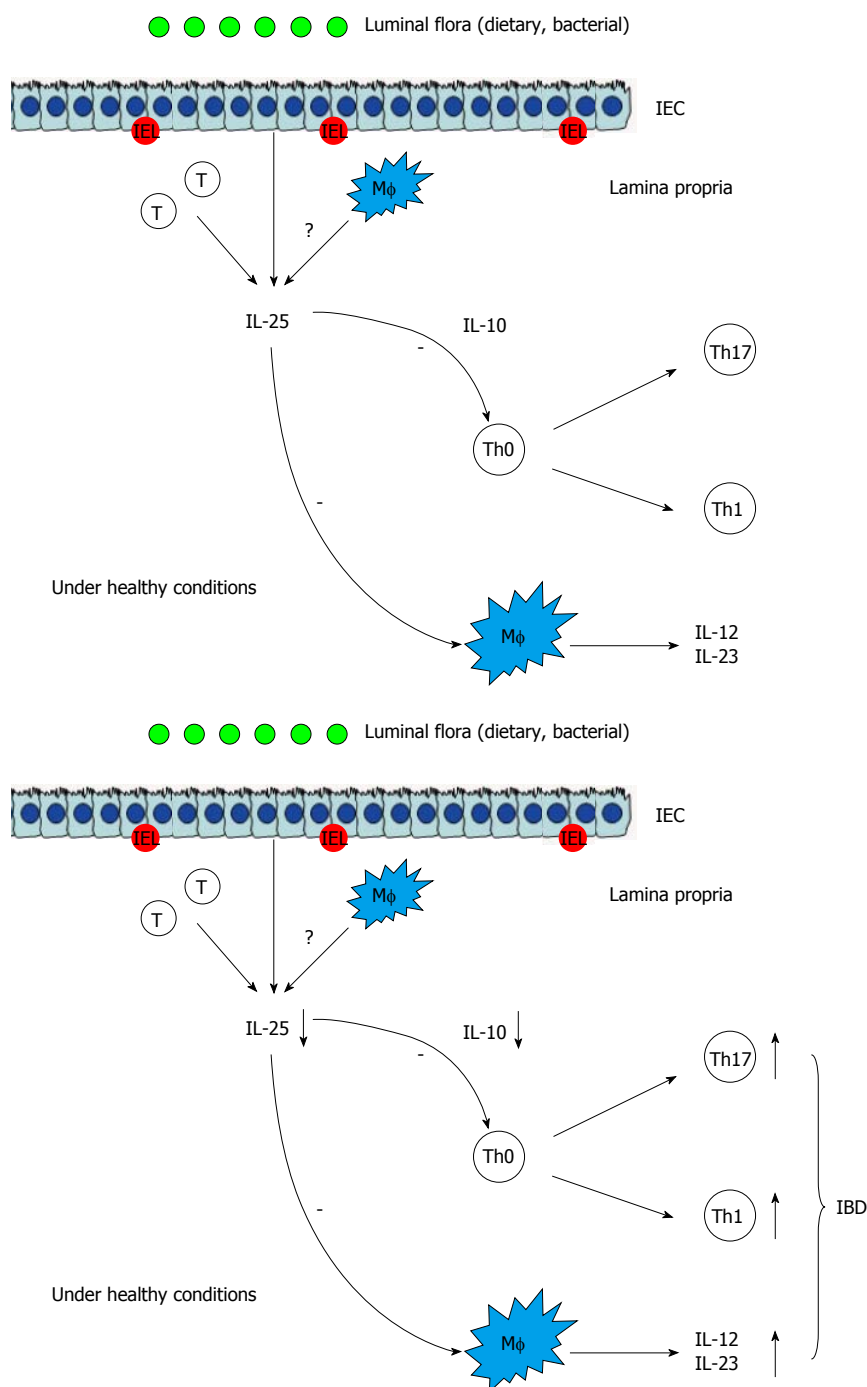


Figure 4 Inhibitory role of interleukin-25 in intestinal mucosa. IEL: Intestinal epithelial lymphocyte; IL: Interleukin; Th: T helper; Treg: T regulator cell; CTL: Cytotoxic T lymphocyte; Mφ: Macrophages; IEC: Intestinal epithelial cells; IBD: Inflammatory bowel disease.

in sites of chronic intestinal inflammation, high levels of IL-23 may activate their full pathogenic and antibacterial functions. Recently, Secukinumab (an IL-17A antibody), Brodalumab (an IL-17 receptor antibody) and two small-molecule drugs (Vidofludimus and Tofacitinib) are used in clinical trials for IBD patients, which inhibit IL-17 as part of their overall pharmacological profiles^[44].

IL-27 AND IL-35: NEWER MEMBERS OF THE IL-12 FAMILY

The IL-12 family is made up of secreted heterodimers

with some overlapping usage between family members. IL-12 (p35/p40) and IL-23 (p19/p40) are the best-known members of the IL-12 family. Other members include IL-27 (EBi3/p28) and IL-35 (EBi3/p35). Like the IL-17 family, however, a critical issue is whether these molecules are pathogenic or protective in the gut. IL-27, produced mostly by myeloid cells, is present at increased concentrations in IBD mucosa. If T cells are taken from mice deficient in the IL-27R and injected into lymphopenic mice, they induce significantly less colitis than wild-type T cells, clearly suggesting that IL-27 is pathogenic in this model. IL-27R-null mice are also less susceptible to DSS-induced

colitis, again suggesting that IL-27 is a proinflammatory mediator. In marked contrast, however, treating mice with established TNBS-induced colitis with IL-27 reduces disease and cytokine production. In humans, IL-27 has also been observed to reduce proinflammatory cytokine production in M ϕ activated with TNF^[45].

MICRORNA AND THE INTESTINAL IMMUNE HOMEOSTASIS

MicroRNA (miR) is an emerging group of short, non-coding RNAs that play an important role in regulating expression of classical genes at the post-transcriptional level. miR regulates cell proliferation, apoptosis, growth, cell differentiation, metabolism and other processes. Recently, miR in intestinal epithelial cells has been found to regulate intestinal mucosal barrier function through its important effect on intestinal epithelial cell proliferation and differentiation. Moreover, differential expression of miR in immune cells within the intestinal mucosa affects the intestinal immune homeostasis^[46,47].

In recent years, evidence has suggested that intestinal epithelial miR expression is closely related with the incidence and development of IBD^[46]. The expression of miR-192, miR-375 and miR-422b is significantly decreased in inflamed mucosa of patients with active UC, while miR-16, miR-21 and let-7 expression is significantly increased. miR-19b and miR-629 are significantly decreased in patients with active CD inflammation within the intestinal mucosa, while miR-23b, miR-106 and miR-191 are significantly increased. The abnormal miR expression will affect translation process of its corresponding target gene mRNAs, regulate gene expression and thus participate in inflammatory injury of intestinal mucosa in IBD. A recent study by Coskun *et al*^[48] provides the first evidence that miR-20b, miR-98, miR-125b-1*, and let-7e* are deregulated in patients with UC.

We have also investigated the expressions of miR10a in IBD and found that the expression of miR10a is decreased in inflamed intestinal mucosa of IBD patients. Furthermore, we have also found that TNF inhibits miR10a expression, while blockade TNF with anti-TNF mAb markedly enhances miR10a expression in the intestinal mucosa (unpublished data). Our findings, together with previous data showing that miR10a could block intestinal inflammation in mice and reduce the differentiation Th1 and Th17^[49], further prove that miR10a is involved in intestinal mucosal inflammatory response, and that targeted therapy may be beneficial for human IBD.

ROLE OF REGULATORY B CELLS

B cells are a source of inhibitory cytokines such as IL-10 and TGF- β . Depending on the signals B cells receive, pro- or antiinflammatory cytokines can be produced, and the shift towards an inflammatory or a protective/suppressive response will be induced. Specific B cell subset found to affect autoreactive responses and suggested to

have a regulatory role in autoimmune diseases is B-regulatory cells (Bregs). CD19⁺CD25⁺ B cells were the first subset of human B cells previously suggested to have a regulatory role. CD19⁺CD25⁺ B cells contribute up to 30% of all peripheral blood B cells in mice and can effectively present peptides to helper T cells. In humans, only B cells expressing high levels of CD25 (BCD25⁺) seem capable of acting as Bregs with abundant TGF- β production. However, it remains unknown how to identify Bregs with membrane markers or transcription factors. Several signals like B cell receptor (BCR), CD40 and/or Toll-like receptor (TLRs) may include. These different activation signals (alone or combined) were shown by many studies to increase regulatory functions of Bregs^[50].

Bacterial molecules have been used to stimulate Bregs. In mice, splenic B cells stimulated *ex vivo* by bacteria acquire the CD5⁺ CD1d⁺ phenotype, which is characterized by high level IL-10 expression and being capable of markedly suppressing the activity of experimental IBD. The protective subset (contributing 1% to 2% of all splenic B cells) is composed of CD5⁺CD1d(high) B cells activated *via* the TLR2/4 pathway by bacterial antigens in the gut flora. Investigations into the influence of the gut microbiota on the balance between effector B cells and Bregs may open up new therapeutic possibilities in IBD^[51,52].

ROLE AND FUNCTIONAL ALTERATIONS OF THE INNATE IMMUNITY

Innate immunity prevents pathogens from entering and spreading within the body. The intestinal innate immune system involves three lines of defense: the mucus layer, epithelium, and lamina propria. Mucus is the first line of intestinal defense, and the major constitutive proteins are mucins (MUC), with diverse isotypes in different portions of the gastrointestinal tract. CD patients show decreased MUC1 and MUC4 levels in the ileum, while MUC2, MUC5AC, MUC5B, MUC6, and MUC7 are undetectable in lesions. UC patients also show decreased MUC2 expression. As the second line of defense, the intestinal epithelium is composed of a monolayer of fast replicating polarized cells: enterocytes, goblet cells, and enteroendocrine cells. All are bound together through tight junctions that separate the body from intestinal lumen components. The membrane TLRs and cytosolic nucleotide oligomerization domain receptor (NOD) are the most important among intestinal pathogen recognition receptors. NOD2 has also been described as a negative regulator of TLR2-mediated IL-12 secretion. Some NOD2 mutations have been described in CD patients, which are associated with decreased defensin secretion by ileal mucosa Paneth cells.

Lamina propria as a third line of intestinal defense contains innate and immune cells. In IBD patients, DCs promote a robust recognition of bacterial products that might cause an immune response to commensal bacteria, provoking a loss of intestinal tolerance. Intestinal macro-

phages from IBD patients have lost the ability to maintain tolerance, mainly through increased surface CD14 content and NF- κ B transcription pathway activity, which might induce increased peripheral macrophage recruitment. Increased cytotoxic activity and elevated NK cell counts are also found in IBD patients. Crucial molecules in NK cells, such as IL-15, IL-21 and IL-23, and their cognate receptors, are elevated in the intestinal mucosa of UC patients. Moreover, in IBD the IEL activation increases, resulting in elevated production of IFN- γ , TNF and IL-2, which is associated with increased IL-23^[53,54].

Deregulated mucin expression in IBD patients might be due to the cytokine imbalance that characterizes these diseases. These molecules stimulate various transcription factor pathways, such as JAK/STAT and NF- κ B, and induce mucin secretion. The combination of TNF and IFN- γ could also decrease claudin-3, claudin-5 and claudin-7 expression, with a marked increase in paracellular permeability in rat colon. Moreover, TNF, IL-6 and IFN- γ increase apoptosis and monolayer permeability in HT-29/B6 cells. These cytokines also inhibit the wound-healing in HT-29/B6 cells. Increased apoptosis and delayed woundhealing of epithelial cells would augment monolayer permeability, and damage the epithelial barrier function^[54,55].

CONCLUSION

The exact etiology of IBD is still not completely understood, and increasing data have demonstrated that these conditions occur through an inappropriate immune response to a subset of commensal enteric bacteria in a genetically susceptible host, with disease initiated by environmental triggers. Dysfunction of the mucosal immune system evokes intestinal inflammation through the activation of both innate and acquired immunity in the gut. Among these, T cell activation and Teff/Treg imbalance play an important role in the process of inflammation. Understanding of immunopathogenesis of IBD will help us to find new ideas for diagnosis and treatment.

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Liquid biopsy of gastric cancer patients: Circulating tumor cells and cell-free nucleic acids

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Abstract

To improve the clinical outcomes of cancer patients, early detection and accurate monitoring of diseases are necessary. Numerous genetic and epigenetic alterations contribute to oncogenesis and cancer progression, and analyses of these changes have been increasingly utilized for diagnostic, prognostic and therapeutic purposes in malignant diseases including gastric cancer (GC). Surgical and/or biopsy specimens are generally used to understand the tumor-associated alterations; however, those approaches cannot always be performed because of their invasive characteristics and may fail to reflect current tumor dynamics and drug sensitivities, which may change during the therapeutic process. Therefore, the importance of developing a non-invasive biomarker with the ability to monitor real-time tumor dynamics should be emphasized. This concept, so called "liquid biopsy", would provide an ideal therapeutic strategy for an individual cancer patient and would facilitate the development of "tailor-made" cancer management programs. In the blood of cancer patients, the presence and potent utilities of circulating tumor cells (CTCs) and

cell-free nucleic acids (cfNAs) such as DNA, mRNA and microRNA have been recognized, and their clinical relevance is attracting considerable attention. In this review, we discuss recent developments in this research field as well as the relevance and future perspectives of CTCs and cfNAs in cancer patients, especially focusing on GC.

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Key words: Gastric cancer; Biomarker; Liquid biopsy; Circulating tumor cells; Cell-free nucleic acids; MicroRNA

Core tip: The potent utilities of circulating tumor cells and cell-free nucleic acids have recently attracted attention toward their clinical application in therapeutic management of cancer patients. The concept of "liquid biopsy" can allow for repeated samplings and real-time monitoring of tumor dynamics in each individual patient and consequently would facilitate the development of "tailor-made" cancer management programs. Before translating this novel diagnostic and prognostic assay into the clinical settings, further large-scale studies with well-established methods are required to validate its clinical relevance.

Tsujiura M, Ichikawa D, Konishi H, Komatsu S, Shiozaki A, Otsuji E. Liquid biopsy of gastric cancer patients: Circulating tumor cells and cell-free nucleic acids. *World J Gastroenterol* 2014; 20(12): 3265-3286 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i12/3265.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i12.3265>

INTRODUCTION

Gastric cancer (GC) is the fourth most common cancer

and the second leading cause of cancer-related death in the world^[1]. Although recent improvements in diagnostic techniques and peri-operative management have resulted in an increase in the early detection of GC and a decrease in its mortality in the past decades, a total of 986600 new GC cases and 738000 deaths are estimated to have occurred in 2008 worldwide^[1]. Several factors seem to restrict diagnostic and therapeutic strategy for treatment of GC and, consequently, to incur the insufficient survival rate: (1) a lack of satisfactory diagnostic assays for early detection of GC; (2) an absence of valuable prognostic indicators; (3) the insufficient effectiveness of current treatments including surgery and chemotherapy for GC patients with advanced stages; and (4) poorly understood mechanisms of tumor progression and resistance to treatments, and a consequent deficiency of targeted therapy. Therefore, the importance of developing useful diagnostic and monitoring tools should be emphasized to improve the clinical outcome of patients with GC.

In the past few decades, numerous studies have demonstrated the potential utility of blood-based biomarkers such as circulating tumor cells (CTCs) and cell-free nucleic acids (cfNAs)^[2-5]. These promising markers are considered to possess great potential and could facilitate therapeutic strategies for cancer including the following: early detection of diseases, predication of prognostic outcome, monitoring of tumor dynamics and development of novel targeted treatments.

Generally, tumor-linked genetic alterations are investigated using tissue samples from surgical or biopsy specimens. These procedures cannot be conducted routinely owing to their invasive nature especially in recurrent and/or metastatic cases with anatomical and/or clinical difficulties. Moreover, a result acquired from a single biopsy can provide only spatiotemporally restricted information and may fail to reflect its heterogeneity and inconsistent tumor characteristics. Detecting CTCs and cfNAs could serve as a “liquid biopsy” for cancer patients, which would be less invasive compared to surgical or endoscopic biopsy and allow us to have repeated samplings and to track the current status of tumor characteristics, such as therapeutic efficiency and resistance. From these viewpoints, the concept of “liquid biopsy” may lead to a better understanding of the genetic landscape in both primary and metastatic lesions as well as the opportunity for tracing genomic evolution.

In this article, we review the historical backgrounds, characterizations and recent developments of both CTCs and cfNAs in cancer research including GC and discuss future perspectives.

BIOLOGY AND DETECTION OF CTCs

In 1869, Ashworth reported the presence of CTCs for the first time in a case of a metastatic cancer patient, in whom cells similar to those in the primary tumors were found in the blood at autopsy^[6]. Since then, various studies have demonstrated the identification and characteriza-

tion of CTCs in peripheral blood of patients with various malignancies, validating Ashworth's previous remarks. Generally, CTCs are considered to appear at very low concentrations in the peripheral blood of cancer patients, usually a single tumor cell in a background of millions of blood cells^[7,8]. Thus, the accurate detection of CTCs with sufficient sensitivity and specificity has been a major technical challenge for researchers (Figure 1).

Techniques for the isolation and enrichment of CTCs

The approaches of CTC isolation/enrichment can be mainly categorized into two groups: (1) physical methods; and (2) biological methods. Isolation based on physical properties does not require the immunological labeling of CTCs because it depends on the characteristics of CTCs, such as size, density, electric charge, migratory capacity and deformability. These methods include density gradient centrifugation, filtration, and dielectrophoresis. Several filtration-based approaches have been developed based on the concept that the majority of CTCs derived from epithelial cancers are generally larger in diameter than other blood cells^[9,10]. However, significant variations in cell size within an individual patient as well as in different types of tumor cells have been reported^[11-13]. Therefore, new approaches using multiple filters have been investigated to resolve those issues and achieve the accurate enrichment of CTCs^[14,15]. While those new approaches are likely to possess great promise in isolating CTCs, further validation studies should be conducted to verify their significance.

Biological methods are another popular approach for the isolation of CTCs, which rely on immunological antibody-based capture of CTCs. In general, this assay involves positive selection with antibodies against tumor-associated antigens, such as epithelial cell adhesion (EpCAM) and cytokeratins (CKs), as well as negative selection with antibodies against the common leukocyte antigen CD45. In particular, EpCAM has been demonstrated to be over expressed and to function as an oncogene in human epithelial cancers including GC^[16-18]. Among several technologies based on antibody-based isolation, the CellSearch system (Veridex) is the most widely used separation system. In this platform, immunomagnetic beads coated with anti-EpCAM antibodies capture CTCs, followed by immunostaining with both positive markers, which are CK8/18/19 for cytoplasmic epithelial markers and 4',6'-diamidino-2-phenylindole hydrochloride for nucleic acids, and a negative marker, leukocyte-specific marker CD45. Accumulating studies have demonstrated the usefulness of the CellSearch system as diagnostic and prognostic indicator in patients with metastatic disease. To date, it is the only technology that has been approved by the United States Food and Drug Administration for the detection of CTCs in the peripheral blood of patients with metastatic breast, prostate and colon cancers^[19-24].

CTCs are generally thought to be quite heterogeneous in both phenotype and genotype, and only a few cells with malignant features could develop into metastatic

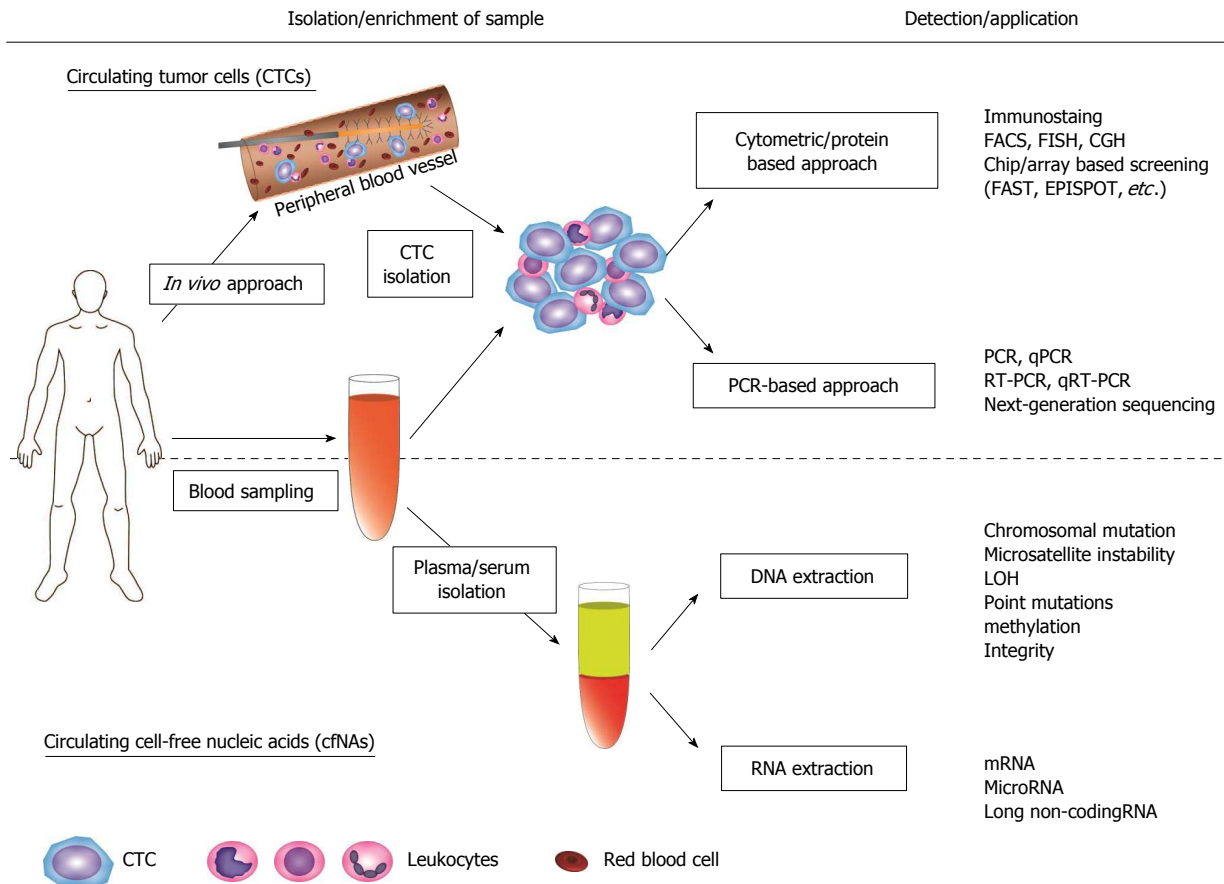


Figure 1 Flow chart of current and potential applications of circulating tumor cell and circulating cell-free nucleic acids technologies. Circulating tumor cells (CTCs): The blood samples from cancer patients are processed through various isolation/enrichment and detection techniques. A new *in vivo* approach allows the enrichment of CTCs directly from a peripheral vein of patients, using a wire functionalized by attachment of epithelial cell adhesion antibodies^[32]. CTCs are usually captured along with contaminating leukocytes. Various detection methods are utilized to detect the rare cell population in the bloodstream. Circulating cell-free nucleic acids (cfNAs): Plasma/serum are generally isolated with centrifuge techniques and subsequently processed for the extraction of specific nucleic acids. Cancer-specific alterations are most commonly analyzed in circulating DNA. Circulating miRNA has attracted increasing attention because of its stability in plasma/serum. Most recently, long non-coding RNA in plasma was also evaluated as a potent biomarker in gastric cancer patients. CGH: Comparative genome hybridization; FACS: Fluorescence activated cell sorter; FISH: Fluorescence *in situ* hybridization; FAST: Fiberoptic array scanning technology; EPISPOT: Epithelial immunospot; qRT-PCR: Quantitative real-time polymerase chain reaction; LOH: Loss of heterozygosity.

tumors. During the journey toward the development of a metastatic lesion, some CTCs might undergo the epithelial-to-mesenchymal transition (EMT), which is characterized by decreased expression of epithelial markers and the acquisition of mesenchymal features^[25]. The EMT has been proposed to be frequently related with cancer aggressiveness and might increase the ability of tumor cells to migrate. Although the identification of EMT-like cancer cells in the bloodstream and its relevance to cancer dissemination is currently under evaluation, assays targeting only epithelial cells may miss the most invasive and potentially significant subpopulation with respect to cancer progression. Therefore, alternative enrichment approaches with different epithelial antigens or negative selection methods aimed to avoid the biased selection of CTC population might be advantageous.

Tumor-specific markers, such as human epidermal growth factor 2 (HER2), prostate-specific antigen (PSA), mucin-1/2 (MUC1/2) and carcinoembryonic antigen (CEA), have also been implemented to capture CTCs

more specifically and adapt to the heterogeneity of CTCs in immunological approaches. More recently, a microfluidic-based device, called the “CTC-Chip”, has been developed for CTC cell detection strategies with a significant increase in yield and purity. Using this new technology, in which whole blood is flowing through chips with automatically optimized flow kinetics, microposts coated with anti-EpCAM antibodies capture CTCs directly from small volumes of blood samples. The CTCs are then stained with secondary antibodies against either CKs or tissue-specific markers, such as PSA in prostate cancer or HER2 in breast cancer, followed by automated scanning of the microposts. Several studies have demonstrated the potent usefulness of this method due to its enhanced sensitivity and specificity, in that the higher number of isolated CTCs facilitates dynamic monitoring during a time course of cancer therapies^[26-28]. Moreover, recent technological progress has allowed for the isolation and analysis of single intact CTCs^[29-31]. These remarkable approaches should have major impacts and further under-

standing of the biology and significance of those heterogeneous populations.

Although most of these technologies have used blood samples *in vitro*, a new revolutionary *in vivo* approach allows the enrichment of CTCs directly from a peripheral vein of patients^[32] (Figure 1). In this system, a structured medical Seldinger guidewire is functionalized with the attachment of EpCAM antibodies. The device is inserted into a peripheral vein, which enables the capture of a large number of CTCs from up to 1.5 L of blood over the duration of 30 min. Despite its potent utility, a large-scale study is required to verify its relevance and to eliminate the possibility of adverse effects.

Techniques for the detection and identification of CTCs

After enrichment of CTCs, identification procedures are conducted to investigate their genetic and biological profiles in detail. Various methodologies for this process have been advocated and developed in the past few decades, ranging from cytometric/protein-based approaches to polymerase chain reaction (PCR)-based approaches. The former approaches involve conventional methods, such as immunostaining for specific markers, fluorescence *in situ* hybridization (FISH) and comparative genomic hybridization, and newly developed methods, such as fiber-optic array scanning technology with high throughput in CTC screening^[33,34] and epithelial immunospot, which can detect proteins secreted from CTCs^[35-37].

PCR-based detection of CTCs has evolved remarkably, especially after the introduction of the quantitative RT-PCR (qRT-PCR) technique, which can minimize possible false-positive results by using a certain “cutoff value” during the analysis process. Identification of appropriate DNA/RNA-based markers expressed by CTCs is considered critical in order to enhance the specificity and reliability of its detection. Therefore, conventional markers for CTCs, such as CKs and CEA, and other diverse markers have been investigated towards their possible clinical application in several malignancies^[38]. CTC-related markers and the introduction of profile analysis including microRNAs (miRNAs) features also might be useful to resolve these issues^[39-41].

CTC detection in patients with GC and its clinical relevance

To date, many researchers have tried to detect CTCs in patients with GC and demonstrated its relevance to biological and oncogenic functions using various approaches. Table 1 represents a summary of previous reports, especially focusing on methodologies, targeted molecules and detection rates. Since its introduction, RT-PCR technology has become the most widely used approach to achieve a satisfactory detection rate despite the extremely low concentration of CTCs in the bloodstream. However, a high sensitivity of RT-PCR may cause an increase in false positive detection even in healthy controls. Therefore, some researchers have utilized multiple detection markers in an mRNA-based assay and suggested its potent usefulness^[42-44].

Of particular note, Wu *et al.*^[44] have developed a sensitive assay using a high-throughput colorimetric membrane array, in which multiple markers, such as human telomerase reverse transcriptase (TERT), cytokeratin 19 (CK19), CEA and MUC1, are measured simultaneously and the combination of four markers serves as a prognostic indicator for overall survival and postoperative recurrence/metastasis in GC. Recently, non-coding RNAs, such as miRNAs and Piwi-interacting RNAs (piRNAs), have been proven to alter their expression in carcinogenesis and tumor progression^[45-47], so these cancer-specific alterations have been reported to be useful for the detection of CTCs in GC^[48-52]. However, some of those reports, in which a mononuclear cell layer was used to isolate total RNA, may not reflect miRNAs originating only from CTCs because the possibility of contamination by leukocyte-originated RNAs cannot be excluded. The presence of miRNAs originating from peripheral blood cells has been demonstrated in the blood of both cancer patients and normal individuals, and furthermore, contamination from those miRNAs has been observed even for circulating cell-free miRNA analysis^[53,54]. Those issues should be addressed before proceeding to clinical practice, and moreover, exhaustive exploration to identify more sensitive miRNA/piRNA-related markers might be desirable to achieve an accurate assay.

Recurrence and metastasis are the most critical factors not only for predicting clinical outcome but also for the quality of life in patients with GC. As summarized in Table 2, accumulating reports have suggested the significance of CTC detection as a prognostic indicator by various approaches, including both the CellSearch System and RT-PCR/qRT-PCR methods. Hiraiwa *et al.*^[55] examined CTCs in 130 gastrointestinal cancer patients involving 44 GC patients using the CellSearch System. Their results demonstrated that the metastatic GC patients with ≥ 2 CTCs ($n = 15$) had a significantly shorter overall survival rate than the metastatic GC patients with < 2 CTCs ($n = 12$) ($P = 0.039$). In a prospective study, Matsusaka *et al.*^[56] also evaluated the relevance of CTCs to chemotherapy and clinical outcome using the CellSearch System. Their results showed that GC patients with ≥ 4 CTCs at 2 and 4 wk after the initiation of chemotherapy had significantly shorter overall survival and progression-free survival in comparison with GC patients with < 4 CTCs, whereas CTC status at baseline (*i.e.*, before the initiation of chemotherapy) had no statistical association with clinical outcomes. These findings may imply the close relationship of CTC status and treatment response.

The majority of the studies using RT-PCR/qRT-PCR methods, which are also widely used for prognosis analysis, have relatively small numbers of cases. Under such circumstances, Mimori *et al.*^[57] focused on one candidate marker, membrane type 1 matrix metalloproteinase (MT1-MMP) mRNA levels, based on results from cDNA microarray analysis, and consequently validated its relevance in a subsequent qRT-PCR based study involving more than 800 GC patients. As a result, MT1-MMP

Table 1 Detection of circulating tumor cells in gastric cancer

Characteristic and number of patients		Control (n)	Detection method		Detection rate/statistic value		Ref.
Pre or post treatment	9 (PB)	4	RT-PCR	CEA mRNA	22.2% 0%	(Pt.) (Ctrl.)	Funaki <i>et al</i> ^[133]
I -IV	20 (PB)	22	RT-PCR	CEA mRNA	35% 0%	(Pt.) (Ctrl.)	Mori <i>et al</i> ^[134]
I -IV	49 (PB) 21 (PV)	50	RT-PCR	CK19 mRNA	0% 0%	(PB) (PV) (Ctrl.)	Aihara <i>et al</i> ^[135]
I -IV	30 (PB)	58	RT-PCR	CK20 mRNA	16.7% 3.4%	(Pt.) (Ctrl.)	Soeth <i>et al</i> ^[136]
Inoperable/ metastatic	34 (PB)	33	RT-PCR	CK19 mRNA	20.6% 0%	(Pt.) (Ctrl.)	Yeh <i>et al</i> ^[137]
I, III, IV	35 (PB)	9	RT-PCR	CEA mRNA	45.7% 0%	(Pt.) (Ctrl.)	Noh <i>et al</i> ^[138]
I -IV	52 (PB)	14	RT-PCR	CK19 mRNA	9.6% 0%	(Pt.) (Ctrl.)	Majima <i>et al</i> ^[139]
				CK20 mRNA	9.6% 1%	(Pt.) (Ctrl.)	
I -IV	41 (PB) (36 with curative surgery) (5 with inoperable)		RT-PCR	CEA mRNA	22.2% 33.3% 80%	(before curative surgery) (during curative surgery) (inoperable Pt.)	Nishida <i>et al</i> ^[60]
I -IV	57 (PA) 49 (PV) 51 (SVC)	30	RT-PCR	CEA mRNA	PA: 17.5% PV: 18.4% SVC: 21.6%		Miyazono <i>et al</i> ^[59]
					8.8% 33.3% 0%	(before surgery) (during surgery) (Ctrl.)	
EGC, III (paired, after surgery during surgery and follow-up)	29 (PB)	15	RT-PCR	CEA mRNA	EGC: 22.2% IIIa: 20% IIIb: 26.7% Total: 24.1% EGC: 22.2% IIIa: 20% IIIb: 34.4% Total: 34.4%	(after surgery) (during follow-up)	At least either one positive in PA/PV/SVC Noh <i>et al</i> ^[140]
					0%	(Ctrl.)	
I -IV (during surgery)	106 (PB)		RT-PCR	CEA mRNA	40.6%		Sumikura <i>et al</i> ^[141]
I -IV	41 (PB)	10	RT-PCR	CEA mRNA	24.4% 0%	(Pt.) (Ctrl.)	Koike <i>et al</i> ^[142]
I -IV	46 (PB) (18 EGJ cancer)	10 (with benign GI disease)	qRT-PCR	CK20 mRNA	27.8%	(Pt. with EGJ cancer)	Friederichs <i>et al</i> ^[143]
			Oncoquick density gradient centrifugation		21.4% 0% 1%	(Pt. with GC) (with benign GI disease) (tumor-free Ctrl.)	
I -IV	59 (PB)	15	qRT-PCR	CEA mRNA	0% 45.8% 0%	(before surgery) (after surgery) (Ctrl.)	Ikeguchi <i>et al</i> ^[58]
I -IV	70 (PB) (41 with curative resection) (29 with residual tumor)		RT-PCR	CK20 mRNA	36.6% 44.8% Total: 40%	(Pt. with curative resection) (Pt. with residual tumor)	Illert <i>et al</i> ^[144]
I -III	46 (PB) (with curative resection) (paired, before and after surgery)	13	RT-PCR	CEA mRNA	52.2% 19.6% 0%	(before surgery) (after surgery) (Ctrl.)	Seo <i>et al</i> ^[145]
I -IV	52 (PB)	36	RT-PCR	c-Met mRNA	61.5% 5.6% 71.2% 8.3%	(Pt.) (Ctrl.) (Pt.) (Ctrl.)	Uen <i>et al</i> ^[142]
				MUC1 mRNA			
I -IV	42 (PB)	30	RT-PCR	hTERT mRNA	61.9% 0% 69.0% 3.3%	(Pt.) (Ctrl.) (Pt.) (Ctrl.)	Wu <i>et al</i> ^[143]
				CK19 mRNA			
				CK20 mRNA			

				CEA mRNA	3.3%	(Ctrl.)	
					78.6%	(Pt.)	
					0%	(Ctrl.)	
I -IV	64 (PB)	80	MAH	hTERT mRNA	81.3%		Wu <i>et al</i> ^[144]
				CK19 mRNA	78.1%		
				CK20 mRNA	82.8%		
				MUC1 mRNA	84.4%		
					No detection in controls		
I -IV	32 (PB)		FACS/ICC	CK8/18/19	21.9%	(before chemotherapy)	Kolodziejczyk <i>et al</i> ^[146]
(paired, before and after chemotherapy)					15.6%	(after chemotherapy)	
I -IV	57 (PB)		FACS/ICC	CK8/18/19	54.4%	(before surgery)	Pituch-
(before surgery)	52 (PB)				21.2%	(after surgery)	Noworolska
(after surgery)	56 (TDB)				26.8%	(TDB sample)	<i>et al</i> ^[61]
I -IV	52 (PB)	20	qRT-PCR	CEA mRNA	5.0%	(before surgery)	Tani <i>et al</i> ^[147]
	(40 pre-o-pe)				16.7%	(after surgery)	
I -IV	41 (PB)	41	Cell search system	EpCAM	14.3%	(Nonmetastatic GC)	Hiraiwa <i>et al</i> ^[155]
				CK8/18/19	55.6%	(metastatic GC)	
					0%	(Ctrl.)	
I -IV	101 (PB)	14	qRT-PCR	CK19 mRNA↑	$P = 0.0127$	Curative resection ($n = 69$)	Koga <i>et al</i> ^[148]
	(69 with curative o-pe)				$P = 0.0087$	<i>vs</i> Ctrl. ($n = 14$)	
	(32 with non-curative o-pe)			CK20 mRNA↑	$P = 0.0022$	Non-curative resection ($n = 32$) <i>vs</i> Ctrl. ($n = 14$)	
						Non-curative resection ($n = 32$) <i>vs</i> Ctrl. ($n = 14$)	
I -IV	810 (PB)	29	RT-PCR	MT1-MMP mRNA	22.8%		Mimori <i>et al</i> ^[57]
					No data for Ctrl.		
I -IV	55 (PB)	86	RT-PCR	Survivin	45.5%	(Pt.)	Yie <i>et al</i> ^[149]
			ELISA	mRNA↑			
					AUC = 0.772	Pt. ($n = 55$) <i>vs</i> Ctrl. ($n = 86$)	
I -IV	70 (PB)	20	qRT-PCR	CEA mRNA	45.7%		Bertazza <i>et al</i> ^[150]
				CK19 mRNA	97.1%		
				VEGF mRNA	38.6%		
				Survivin mRNA	98.6%		
					(Control samples were used the calibrator source)		
I -IV	846 (PB)	25	qRT-PCR	uPAR mRNA↑	404/846 47.8%		Kita <i>et al</i> ^[151]
					$P < 0.0001$	Pt. ($n = 846$) <i>vs</i> Ctrl. ($n = 25$)	
Advanced	52 (PB)		Cell search system	EpCAM	32.7%	(baseline)	Matsusaka <i>et al</i> ^[156]
(paired, before and during chemotherapy)				CK8/18/19	13.7%	(2 wk after chemotherapy)	
					18.8%	(4 wk after chemotherapy)	
I -IV	123 (PB)	30	qRT-PCR	CEA mRNA	36.6%	(Pt.)	Qiu <i>et al</i> ^[152]
					30%	(Ctrl.)	
I -IV	30 (PB)		qRT-PCR	CK18 mRNA	I / II : 81.8%		Saad <i>et al</i> ^[153]
(after curative surgery)					III / IV : 31.6%		
N/A	90 (PB)			miR-106a↑	Total: 50%	Pre-o-pe <i>vs</i> Ctrl.	Zhou <i>et al</i> ^[48]
	(90 before surgery)				$P = 0.006$		
	(41 preoperative)			miR-17↑	AUC = 0.684	Post-o-pe <i>vs</i> Ctrl.	
	(49 postoperative)				$P = 0.016$	Pre-o-pe <i>vs</i> Ctrl.	
					$P = 0.001$		
					AUC = 0.743		
					$P = 0.019$	Post-o-pe <i>vs</i> Ctrl.	
I -IV	95 (PB)	21	qRT-PCR	B7-H3 mRNA↑	50.5%		Arigami <i>et al</i> ^[154]
					$P < 0.0001$	Pt. ($n = 95$) <i>vs</i> Ctrl. ($n = 21$)	
					AUC = 0.86		
I -IV	98 (PB)	30	RT-PCR	Survivin	I / II : 25%		Cao <i>et al</i> ^[155]
			ELISA	mRNA			
					III / IV : 56.1%		
					I -IV : 45.9%		
I -IV	93 (PB)	32	qRT-PCR	piR-651↓	$P < 0.001$	Pt. ($n = 93$) <i>vs</i> Ctrl. ($n = 32$)	Cui <i>et al</i> ^[49]
	(42 pre-o-pe)				AUC = 0.841		
	(51 post-o-pe)			piR-823↓	$P < 0.001$		
					AUC = 0.822		
II -IV	35 (PB)	50	qRT-PCR	CEA mRNA	22.9%	(Pt.)	Dardaei <i>et al</i> ^[156]

				CK20 mRNA	37.1%	(Pt.)	
				TFF1 mRNA	31.4%	(Pt.)	
				MUC2 mRNA	22.9%	(Pt.)	
				No detection in controls			
I-IV	53 (PB)	20	qRT-PCR	miR-21↑	$P < 0.0001$	Pt. ($n = 53$) vs Ctrl. ($n = 20$)	Zheng <i>et al.</i> ^[50]
					AUC = 0.853		
I-IV	52 (PB)	15	qRT-PCR	miR-200c↑	$P = 0.018$	Pt. ($n = 52$) vs Ctrl. ($n = 15$)	Valladares-Ayerbes <i>et al.</i> ^[51]
					AUC = 0.715		
I-IV	40 (PB)	17	qRT-PCR	miR-421↑	$P < 0.01$	Pt. ($n = 40$) vs Ctrl. ($n = 17$)	Zhou <i>et al.</i> ^[52]
					AUC = 0.773		

AUC: Area under the receiver operating characteristic curve; BM: Bone marrow; EGC: Early gastric cancer; EGJ: Esophagogastric junction; GI: Gastrointestinal; MAH: Membrane-array hybridization; PA: Peripheral artery; PB: Peripheral blood; PV: Portal vein; SVC: Superior vena cava; TDB: Tumor-draining blood; N/A: Not available; qRT-PCR: Quantitative real-time polymerase chain reaction; FACS: Fluorescence activated cell sorter; ICC: Intracellular cytokine flow cytometry.

mRNA levels in peripheral blood were indicated to be an independent factor for determining recurrence and distant metastasis of GC ($P = 0.0018$).

Intriguingly, some groups have reported time-dependent changes in the detection rate of CTCs during the peri-operative time course^[58-61]. Those changes may imply the possibility of monitoring the tumor dynamics; however, the biological and clinical meaning of CTCs still remains unknown and controversial. In fact, incompatible events, including both increase and decrease in CTC detection rates during surgical maneuvers, have been proposed so far^[58-61]. This discrepancy might be partially explained by a wide variety of measurement parameters, from the methodology itself to targeted markers, patient background/properties and sample conditions.

In summary, recent technological advances have provided considerable progress and interest in the detection of CTCs in various cancers, including GC. Although previous studies have shown a potent usefulness of CTC detection as a novel diagnostic and prognostic assay in cancer patients, little remains known about the biological features and fundamental roles of these cells. Detailed characterization of CTCs and well-designed experiments should resolve current underlying issues and provide the opportunity for clinical impact in cancer therapy.

BIOLOGY AND DETECTION OF CELL-FREE NUCLEIC ACIDS

The study of cfNAs has a considerably long history since it was first reported in 1948 by Mandel and Meitais^[62], who successfully detected nucleic acids in human plasma. Unfortunately, their work attracted little attention at that time owing to a lack of sufficient understanding of that innovative concept. Regarding malignant disease, in 1977, Leon *et al.*^[63] first reported the presence of cell-free DNA (cfDNA) in the serum of cancer patients. Furthermore, they also mentioned its potent function as a clinical indicator, showing decreased cfDNA levels in response to radiotherapy. In 1989, Vasioukhin *et al.*^[64] successfully detected cfDNA with neoplastic characteristics and proposed the first evidence suggesting that tumors can shed DNA into the circulation. This hypothesis was further strengthened by two studies in 1994, in

which NRAS mutations in the plasma of patients with myelodysplastic syndrome or acute myelogenous leukemia, and KRAS mutations in the plasma or serum of patients with pancreatic cancer^[65], were detected. Those findings opened up a new field in the exploration of circulating nucleic acids, and many meritorious studies have demonstrated the biological function of cfNAs and their potential as novel biomarkers regarding DNA, mRNA and miRNAs (Figure 1).

In regard to the origin of circulating nucleic acids, two main potent release mechanisms, called “passive” and “active”, are advocated to date. The passive mechanism involves the release of nucleic acids originated from apoptotic and necrotic cells into the bloodstream. Macrophages and phagocytes play an important role in phagocytosis of necrotic and apoptotic cells and can release digested nucleic acids into the microenvironment^[66,67]. In contrast, it is reported that fragments of cellular nucleic acids can be actively released^[68,69]. Although the active secretion into the circulation remains enigmatic, one potential explanation is that cancer cells would release nucleic acids to transform the targeted recipient cells at distant locations^[70-72]. In addition to those two mechanisms, cfNAs might be released by CTCs, however, there appears to be a huge gap between the amount of cfNAs and the rarity of CTCs in the bloodstream as described in the previous section. Thus, this hypothesis has been controversial so far.

Circulating cell-free DNA in plasma/serum

The study of circulating cfDNA in the plasma/serum involves the measurement of the total volume of circulating DNA as well as the detection of cancer-related genetic/epigenetic aberrations, which include microsatellite instability, loss of heterozygosity, genetic polymorphisms, point mutations, methylation, deletion/amplification/translocation of chromosome and integrity (*i.e.*, the ratio of longer DNA fragment to shorter one based on the different cleavage process between apoptosis and necrosis^[73]). The latter approach is generally recognized to be able to cover a wider range of oncogenic alterations in various cancers and to possess more potent application in the clinical setting than the former one, partly because cfDNA can be released into the bloodstream and is

Table 2 Prognostic value of circulating tumor cells in gastric cancer

Characteristic and number of patients		Detection method		Statistic value			Ref.
(non-responsive to chemotherapy)	17	RT-PCR	CK19 mRNA	OS	$P = 0.014$	CK19 (+) <i>vs</i> (-)	Yeh <i>et al</i> ^[137]
I -IV	57	RT-PCR	CEA mRNA	Liver metastasis recurrence	$P = 0.03$	CEA (+) <i>vs</i> (-)	(a) Miyazono <i>et al</i> ^[159]
I -IV	106	RT-PCR	CEA mRNA	Recurrence/ metastasis	$P = 0.02$	CEA (+) <i>vs</i> (-)	(a) Sumikura <i>et al</i> ^[141]
I -IV	46	qRT-PCR	CK20 mRNA	2-year-survival	$P < 0.05$	CK20 (+) <i>vs</i> (-)	N/A Friederichs <i>et al</i> ^[143]
I -IV	41	RT-PCR	CK20 mRNA	OS	$P = 0.0363$	CK20 (+) <i>vs</i> (-)	(b) Illert <i>et al</i> ^[144]
(with curative resection)	46	RT-PCR	CEA mRNA	Recurrence	$P \leq 0.00022$	CEA after sugery (+) <i>vs</i> (-)	(a) Seo <i>et al</i> ^[145]
I -IV	52	RT-PCR	C-Met mRNA	Recurrence	$P = 0.015$		(c) Uen <i>et al</i> ^[142]
			MUC1 mRNA	OS	$P = 0.0178$	C-Met (+) <i>vs</i> (-)	(b)
I -IV	42	RT-PCR	CEA mRNA	OS	$P = 0.0352$	MUC1 (+) <i>vs</i> (-)	(b)
				Recurrence/ metastasis	$P = 0.032$	CEA (+) <i>vs</i> (-)	(c) Wu <i>et al</i> ^[143]
I -IV	64	MAH	hTERT/CK19/ CEA/MUC1	Recurrence/ metastasis	$P = 0.009$	All marker (+) <i>vs</i> the others	(c) Wu <i>et al</i> ^[144]
				OS	$P = 0.0223$		(b)
Metastatic	27	CellSearch System	EpCAM	OS	$P = 0.039$	CTC ≥ 2 <i>vs</i> < 2	(b) Hiraiwa <i>et al</i> ^[155]
I -IV	69	qRT-PCR	CK19 mRNA	OS	$P = 0.0347$	CK19 (+) <i>vs</i> (-)	(b) Koga <i>et al</i> ^[148]
(with curative resection)			CK20 mRNA	OS	$P = 0.049$	CK20 (+) <i>vs</i> (-)	(b)
I -IV	810	RT-PCR	MT1-MMP	Recurrence/ metastasis	$P = 0.0018$	MT1-MMP (+) <i>vs</i> (-)	(c) Mimori <i>et al</i> ^[157]
I -IV	55	RT-PCR	Survivin mRNA	RFS	$P = 0.026$	Survivin (+) <i>vs</i> (-)	(b) Yie <i>et al</i> ^[149]
		ELISA					
I -IV	70	qRT-PCR	Survivin mRNA	OS	$P = 0.026$	Survivin high <i>vs</i> low	(d) Bertazza <i>et al</i> ^[150]
					$P = 0.036$		(b)
					$P < 0.001$		(d)
Advanced	51	Cell search system	EpCAM	PFS (2 wk after chemotherapy)	$P < 0.001$	CTC ≥ 4 <i>vs</i> < 4	(b) Matsusaka <i>et al</i> ^[156]
			CK8/18/19		$P < 0.001$		(d)
(2 wk after chemotherapy)							
	48			OS (2 wk after chemotherapy)	$P < 0.001$		(b)
(4 wk after chemotherapy)					$P < 0.001$		(d)
				PFS (4 wk after chemotherapy)	$P < 0.001$		(b)
					$P < 0.001$		(d)
				OS (4 wk after chemotherapy)	$P < 0.001$		(b)
					$P = 0.004$		(d)
I -IV	123	qRT-PCR	CEA mRNA	Recurrence	$P = 0.001$	CEA (+) <i>vs</i> (-)	(a) Qiu <i>et al</i> ^[152]
				DFS	$P = 0.001$		(b)
					$P = 0.02$		(d)
I -IV	30	qRT-PCR	CK18 mRNA	RFS	$P < 0.001$	CK18 (+) <i>vs</i> (-)	(b) Saad <i>et al</i> ^[153]
(after curative surgery)					$P = 0.04$		(d)
				OS	$P = 0.001$		(b)
					$P = 0.06$		(d)
I -IV	95	qRT-PCR	B7-H3 mRNA	OS	$P = 0.02$	B7-H3 high <i>vs</i> low	(b) Arigami <i>et al</i> ^[154]
					$P = 0.046$		(d)
I -IV	98	RT-PCR	Survivin mRNA	DFS	$P < 0.001$	Survivin (+) <i>vs</i> (-)	(b) Cao <i>et al</i> ^[155]
		ELISA			$P < 0.001$		(d)
I -IV	52	qRT-PCR	miR-200c	OS	$P = 0.016$	miR-200c high <i>vs</i> low	(b) Valladares-Ayerbes <i>et al</i> ^[151]
					$P = 0.028$		(d)
				RFS	$P = 0.044$	miR-200c high <i>vs</i> low	(b)
					$P = 0.028$		(d)

a: χ^2 test/Fisher's exact test; b: Kaplan-Meier survival curves, Log-rank test/Breslow-Wilcoxon test; c: Logistic regression model (multivariate); d: Multivariate Cox proportional hazard regression model. DFS: Disease-free survival; MAH: Membrane-array hybridization; OS: Overall survival; PFS: Progression-free survival; qRT-PCR: Quantitative real-time polymerase chain reaction; RFS: Relapse-free survival.

detectable in the plasma/serum in healthy humans^[69,74]. In fact, numerous reports have demonstrated the detection of genetic and epigenetic alterations in circulating DNA in the plasma/serum in cancer patients^[75-79]. Furthermore, in colorectal cancer, recent two reports clearly demonstrated a correlation between acquired resistance to the anti-EGFR antibody drugs, such as cetuximab and panitumumab, and the emergence of KRAS mutations, which was successfully detected and monitored in the blood of patients under treatment^[80,81]. Misale *et al.*^[81] also indicated the potential of cfDNA to monitor tumor dynamics more sensitively compared to conventional assays, showing that KRAS mutant alleles were confirmed in the blood of a cetuximab-treated patient 10 mo earlier than radiographic examinations. Moreover, Leary *et al.*^[82] have recently analyzed individual tumor-specific DNA translocations in paired solid tumor and circulating cfDNA samples using next-generation sequencing technology and consequently demonstrated the feasibility of personalized biomarkers, enabling a so-called “tailor-made” therapeutic strategy. In summary, moving toward the development and future application in the clinical setting, the accumulated evidence has proven the potent usefulness of cfDNA for the detection of disease as well as for the assessment of residual disease, recurrence, and secondary resistance.

Detection of circulating DNA in patients with GC and its clinical relevance

Previous reports regarding circulating cfDNA in GC patients are summarized in Table 3. Among those reports, a few studies with respect to the concentration of circulating cfDNA are found, in which a housekeeping gene, beta-actin^[83], and a non-coding genomic DNA repeat sequence, ALU^[84], were evaluated. In contrast, the detection of methylated DNA in plasma/serum appears to be the most widely used approach in GC, which was usually investigated by methylation specific-PCR (MSP) or quantitative methylation specific-PCR (qMSP) assays. In 2002, Lee *et al.*^[85] first reported the potent application of detecting methylated DNA of death-associated protein-kinase, E-cadherin, GSTP1, p15 and p16 in the serum of GC patients. Thereafter, technological advances and the exploration of more sensitive and specific genes have provided the accumulated evidence in this field. In detail, comprehensive analyses by methylation CpG island microarray have suggested the possibility of more significant genes for detecting methylated DNA^[86,87]. Most recently, Ling *et al.*^[88] clearly demonstrated the potent usefulness of detecting methylated XAF1 DNA as a diagnostic as well as prognostic biomarker with satisfactory degrees of specificity and sensitivity. Specifically, methylated XAF1 DNA in serum was detected in 69.8% (141/202) of the GC patients and none of the healthy individuals (0/88) with an area under the receiver operating characteristic curve (AUC) of 0.909 in a receiver operating characteristic (ROC) curve analysis for discrimination of the two groups and was significantly correlated with

poorer prognosis in GC ($P < 0.001$, disease-free survival, Kaplan-Meier survival curves, Log-rank test).

Concerning genetic alterations in other types of cancers, the relationships with tumor-specific gene alteration such as HER2 in breast cancer^[89] and adenomatous polyposis coli in colorectal cancer^[75,90] have been revealed even in circulating cfDNA. In GC, Park *et al.*^[91] investigated gene amplification of MYC in the plasma of GC patients and showed that the plasma MYC/GAPDH ratio was significantly higher in the GC patients than that in the healthy controls ($P < 0.001$) and correlated with the tissue MYC/GAPDH ratio ($P = 0.009$), and tissue MYC status by FISH ($P = 0.024$). In contrast, among GC patients with a 2+ or 3+ score in a HER2 IHC assay, Lee *et al.*^[92] reported that no significant association was observed between the HER2 level in plasma and the copy number variation in tumor tissue determined by FISH. Although it is unclear why there was a discrepancy between these two results, it may be partially explained by the inappropriate employment of reference genes and the heterogeneity of GC tissues. The investigation of circulating cfDNA relating to genetic aberration in GC remains in its infancy. Therefore, further evidence is expected to address current controversial issues and develop this field.

Circulating cell-free mRNA in plasma/serum

The presence of RNase in plasma/serum had long been known, and furthermore, the RNase concentration in serum was reported to be elevated in cancer patients in the 1970s^[93,94]. Given that mRNA in plasma/serum might be more fragile than DNA and susceptible to degradation by RNase, it was not clear whether mRNA could exist in plasma/serum with sufficient integrity to allow amplification, although several reports had previously suggested the possible presence of RNA in serum forming a complex with proteolipids^[95,96]. In 1999, two groups reported the successful detection of cell-free RNA such as tyrosinase mRNA in serum of patients with malignant melanoma^[97] and epstein-barr virus-associated RNA associated in plasma of patients with nasopharyngeal carcinoma^[98]. Subsequently, many studies have demonstrated the presence of specific mRNA in plasma/serum and its potent clinical relevance in patients with a variety of cancers^[99-101]. At present, it is considered that mRNA in plasma/serum may be protected from degradation by packaging in secretory membrane vesicles, such as exosomes, microvesicles and multivesicles, which are released from cellular surfaces into the bloodstream^[102,103].

Circulating cell-free miRNA in plasma/serum

In the past decade, circulating cell-free miRNAs in plasma/serum have attracted increasing attention among investigators in various types of research field including oncology. The discovery of miRNA dates back to 1993, when Lee *et al.*^[104] found that a short RNA product encoded by the *lin-4* gene inhibited the translation of its putative target, *lin-14* mRNA, with partial sequence complementarity during a study of *Caenorhabditis elegans* (*C.*

Table 3 Circulating cell-free DNA in gastric cancer

Characteristic and number of patients		Controls (<i>n</i>)	Plasma/serum	Detection method		Detection rate/statistic value		Ref.
Unresectable	198	78 (peptic ulcer)	Serum	Immuno-PCR	MG7-Ag	82.8%	(GC)	Ren <i>et al</i> ^[157]
		118 (chronic gastritis)				7.7%	(peptic ulcer)	
		236 (healthy donors)				5.9%	(chronic gastritis)	
						0.8% <i>P</i> < 0.01	(healthy donors) Correlation with metastasis	
N/A	51	30 (gastritis)	Serum	qPCR	EBV DNA	100%	(Pt. with EBER (+) in primary tumor)	Lo <i>et al</i> ^[158]
		197 (healthy controls)				92.9%	(Pt. with EBER (+) in filtrating lymphocytes)	
						0%	(Pt. with EBER (-) in primary tumor)	
						23.3% 3.6%	(gastritis) (Ctrl.)	
I-IV	54	30	Serum	MSP	DAP-kinase E-cadherin GSTP1 p15 p16	48.1% 57.4% 14.8% 55.6% 51.9%		Lee <i>et al</i> ^[85]
I-IV	60	16	Serum	MSP	p16	No detection in controls 26.1%	With p16 methylation in primarily tumor	
						0%	Without p16 methylation in primarily tumor	
						0%	(Ctrl.)	
I-IV	109	10	Serum	MSP	p16 E-cadherin p16 + E-cadherin	18.3% 23.9% 36.7%		Ichikawa <i>et al</i> ^[160]
I-IV	41	10	Serum	MSP	p16 E-cadherin RARb p16 + E-cadherin + RARb	No detection in controls 22.0% 22.0% 14.6% 24.4%		
I-IV	63	10	Serum	MSP	p16 E-cadherin RARb p16 + E-cadherin + RARb	No detection in controls 27.0% 23.8% 17.5% 50.8%		
I-IV	60	22	Serum	qMSP	APC E-cadherin hMLH1 TIMP3 Four markers combined	No detection in controls 16.7% 13.3% 41.7% 16.7% 55% 13.6%	(Ctrl.)	Leung <i>et al</i> ^[162]
						OS: <i>P</i> = 0.006	Methylation (+) vs (-)	
I-IV	109	10	Serum	MSP	RARb	23.8%		
I-IV	53	21	Plasma	qPCR	p16 + E-cadherin + RARb β -actin (102 bp) β -actin (253 bp) DNA integrity (253 bp/102 bp)	47.7% <i>P</i> = 0.03 <i>P</i> < 0.0001 AUC = 0.75 <i>P</i> = 0.07	Pt. (<i>n</i> = 53) vs Ctrl. (<i>n</i> = 21)	
N/A	4	10	Serum	MSP	RUNX3 p16 RASSF1A CDH1	100% 50% 25% 25%		Tan <i>et al</i> ^[164]

I-IV	52 (40 pre-op) (12 post-op)	20	Serum	MSP	p16 E-cadherin RARb	No detection in controls 9.6% 9.6% 3.8%		Tani <i>et al</i> ^[147]
I-IV	52	50	Serum	p16 + E-cadherin + RARb MSP	p16	23.1% 26.9%	(all Pt.)	Abbaszadegan <i>et al</i> ^[165]
I-IV	20	22	Plasma	Fluorescence -based assay MSP	DNA concentration↑ MGMT	0% $P < 0.005$ 70% 36.4%	(Pt. with p16 methylation in primary tumor) (Ctrl.) Pt. (n = 20) vs Ctrl. (n = 22) (Pt.) (Ctrl.)	Kolesnikova <i>et al</i> ^[166]
I-IV	47	30 (benign gastric disease) 30 (healthy controls)	Serum	MSP	RASSF1A	50% 18.2% 25% 9.1% 24.0%	(Pt.) (Ctrl.) (Pt.) (Ctrl.) (Pt.)	Wang <i>et al</i> ^[167]
I-IV	20	21	Serum	MSP	HSulf-1	3.3% 0% 55%	(benign gastric disease) (healthy controls) (Pt.)	Chen <i>et al</i> ^[168]
I-IV	57	79	Plasma	qPCR	MYC/ GAPDH↑	19.0% $P < 0.001$	(Ctrl.) Pt. (n = 57) vs Ctrl. (n = 79)	Park <i>et al</i> ^[191]
I-IV	65	50	Serum	qMSP	RUNX3	AUC = 0.841 29.2%	(Pt.)	Sakakura <i>et al</i> ^[169]
I-IV	65	40 (benign gastric disease) 20 (healthy controls)	Serum	MSP	DLEC1	10% AUC = 0.8651 33.8%	(Ctrl.) Pt. (n = 65) vs Ctrl. (n = 50) (Pt.)	Zhang <i>et al</i> ^[170]
I-IV	73	20	Serum	qMSP	TFPI2	5% 0% 9.6% 0% $P = 0.004$	(benign gastric disease) (healthy controls) (Pt.) (Ctrl.) Correlation with LN meta.	Hibi <i>et al</i> ^[171]
I-IV	65	80	Plasma	qMSP	SLC19A3	$P = 0.0115$ $P < 0.0001$	Correlation with distant meta. Pt. (n = 45) vs Ctrl. (n = 60)	Ng <i>et al</i> ^[172]
I-IV	46	30 (healthy controls) 46 (benign gastric disease)	Serum	Methylation CpG island microarray MSP	BX141696 WT1 CYP26B1 KCNA4	AUC = 0.82 56.5% 50% 73.9% 67.4%	(Validation 1) Pt. (n = 20) vs Ctrl. (n = 20) (Validation 2)	Zheng <i>et al</i> ^[86]
I-IV	58	30 (healthy controls) 46 (gastric precancerous lesions)	Serum	MeDIP Methylation CpG island microarray MSP	CHRM2 FAM5C MYLK FAM5C + MYLK	31.0% 31.0% $P < 0.001$ 70.7% $P < 0.001$ 77.6%	Pre- vs post-operation Pre- vs post-operation (GC Pt.)	Chen <i>et al</i> ^[87]
						10% 30.4%	(healthy controls) (gastric precancerous lesions)	

I-IV	59	54	Plasma	qPCR	ALU↑	$P < 0.001$	Pt. ($n = 59$) vs Ctrl. ($n = 54$)	(c)	Park <i>et al</i> ^[84]
						AUC = 0.784			
N/A	25	9	Plasma	MSP	ATP4B	64% 0%	(Pt.) (Ctrl.)		Raja <i>et al</i> ^[173]
I-IV	71	21	Serum	qMSP	Vimentin	$P = 0.018$	Pt. ($n = 73$) vs Ctrl. ($n = 21$)	(c)	Shirahata <i>et al</i> ^[174]
Operable	73	20	Serum	MSP	SOX17	58.9% 0%	(Pt.) (Ctrl.)		Balgkouranidou <i>et al</i> ^[175]
I-IV	73		Plasma	qPCR	HER2	OS: $P = 0.049$ 64.4%	Methylation (+) vs (-) Pt. with 2+/3+ score in HER2 IHC assay	(b)	Lee <i>et al</i> ^[92]
Pt. with 2+/3+ score in HER2 IHC assay									
I-IV	202	88	Serum	qMSP	XAF1	69.8% 0%	(Pt.) (Ctrl.)		Ling <i>et al</i> ^[88]
						AUC = 0.909	Pt. ($n = 202$) vs Ctrl. ($n = 88$)		
						DFS: $P < 0.0001$	Methylation (+) vs (-)	(b)	

a: χ^2 test/Fisher's exact test; b: Kaplan-Meier survival curves, Log-rank test/Breslow-Wilcoxon test; c: Unpaired *t*-test/Mann-Whitney *U*-test; d: Unknown statistic method. AUC: Area under the receiver operating characteristic curve; LN: Lymph node; MeDIP: Methylated DNA immunoprecipitation; MSP: Methylation specific-PCR; qMSP: Quantitative methylation specific-PCR. qRT-PCR: Quantitative real-time polymerase chain reaction; N/A: Not available; GC: Gastric cancer; EBV: Epstein-barr virus; DAP: Death-associated protein; APC: Adenomatous polyposis coli; OS: Overall survival; HER: Human epidermal growth factor.

elegans) development. In 2000, a second miRNA, *let-7*, was identified to repress the functions of multiple mRNAs in *C. elegans*^[105]. Subsequently, *let-7* was found to be widely conserved across species, implying the ubiquitous roles of miRNAs^[106]. Since then, accumulated research has revealed their biological features in a variety of diseases.

In summary, miRNAs are a group of noncoding small RNAs, whose mature form generally consists of 19-25 nucleotides. miRNAs are involved in post-translational regulation of gene expression by inhibiting stability and translation of mRNAs^[107]. To date, more than 1800 miRNAs have been characterized in *Homo sapiens* according to the miRNA database (miRBase), and the number of listed miRNAs is increasing. It is suggested that one miRNA can regulate multiple different mRNAs, and conversely one mRNA can be regulated by multiple miRNAs^[108,109].

Concerning malignant diseases, miRNAs have been demonstrated to play essential roles in cell proliferation, cell differentiation, apoptosis, EMT, and metastasis^[45,46]. Numerous studies have proven the aberrant expression of miRNA and its critical characteristics including both oncogenic and tumor suppressive functions in various cancers. Those findings have motivated us to accelerate this promising field to the next stage, such as development of miRNA-based biomarkers and therapies^[110,111].

In 2008, the successful detection of circulating miRNAs and their significance in malignant diseases were first reported by several groups^[112-115]. Notably, Mitchell *et al*^[112] and Chen *et al*^[113] clearly demonstrated the biological features and potent utility of circulating miRNA, showing their high stability and reproducibility with resistance to endogenous/exogenous RNase, prolonged incubation at room temperature, extreme pH conditions and multiple freeze-thawing processes. The stability of miRNA in plasma/serum, likely greater than that of mRNA, could be explained by some protective mechanisms, which involve packaging in secretory particles (apoptotic bodies,

exosomes *etc.*)^[72,116] and binding to certain proteins (Argonate 2, High-density Lipoproteins, *etc.*)^[117-119]. Furthermore, secretory vesicles including miRNAs have been shown to be able to function as intercellular transmitters^[72,116,120], suggesting that circulating miRNAs in plasma/serum possess various roles in cancer development and metastasis. Those findings have provided new insight into the screening and monitoring of cancer patients, and emerging evidence has suggested the promising potential of circulating miRNA as a novel and non-invasive biomarker in clinical practice^[121,122].

Detection of circulating cell-free RNA in patients with GC and its clinical relevance

As summarized in Table 4, the number of previous reports regarding circulating mRNA in GC patients is small compared with those regarding other types of cancers and with those concerning circulating DNA in GC. However, as Kang *et al*^[123] recently reported that the detection of plasma hTERT mRNA can serve as a potential marker for diagnosis and prognosis of GC patients, increased insight and evidence about circulating mRNA might facilitate the development of this field.

Since the potent utility of determining miRNAs in the plasma of GC patients was first reported by our group in 2010^[124], many studies have demonstrated the significance of circulating miRNAs as novel biomarkers (Table 5). Still, the immaturity of the field has led to several issues concerning its actual introduction in clinical settings. To date, there has been no consensus regarding how inter- and intra-individual variations can affect the results, which sample (*i.e.*, plasma or serum) is more favorable for measuring circulating miRNA, or which molecule is the most appropriate for the sensitive detection and endogenous controls. Moreover, as mentioned before, one miRNA can regulate multiple mRNAs and the numbers of discovered miRNAs and targeted mRNAs are still

Table 4 Circulating cell-free mRNA in gastric cancer

Characteristic and number of patients	Controls (n)	Plasma/serum	Detection method	Detection rate/statistic value	Ref.
I-IV (40 preoperative) (12 postoperative)	20	Plasma	qRT-PCR hTERT MUC1 hTERT + MUC1 hTERT MUC1 hTERT + MUC1	7.5% 1% 15% 16.7% 8.3% 16.7% (preoperative) (postoperative)	Tani <i>et al</i> ^[147]
I-IV [paired, before and after surgery (n = 69)]	42	Plasma	qRT-PCR CXCR4↑ Bmi-1↑	No detection in controls 41.6% 23.2% 21.4% [before surgery (n = 89)] [after surgery (n = 69)] [Ctrl. (n = 42)] P < 0.05 Before surgery (n = 89) vs Ctrl. (n = 42) 57.3% 43.5% 28.6% [before surgery (n = 89)] [after surgery (n = 69)] [Ctrl. (n = 42)] P < 0.05 Before surgery (n = 89) vs Ctrl. (n = 42) P < 0.05 Before (n = 89) vs after (n = 69) surgery (a) (a) (a)	Xu <i>et al</i> ^[176]
I-IV	118	40 (gastritis) 58 (healthy controls)	Plasma qRT-PCR hTERT mRNA↑	P < 0.05 GC (n = 118) vs gastritis (n = 40) P < 0.05 GC (n = 118) vs Ctrl. (n = 58) AUC = 0.891 DFS: P < 0.001 DFS: P = 0.001 OS: P < 0.001 OS: P < 0.001 GC (n = 118) vs Ctrl. (n = 58) (b) (c) (b) (c)	Kang <i>et al</i> ^[123]

a: Mann-Whitney *U*-test; b: Kaplan-Meier survival curves, Log-rank test/Breslow-Wilcoxon test; c: Multivariate Cox proportional hazard regression model. AUC: Area under the receiver operating characteristic curve; DFS: Disease-free survival; OS: Overall survival; qRT-PCR: Quantitative real-time polymerase chain reaction; hTERT: Human telomerase reverse transcriptase; MUC1: Mucin-1.

increasing owing to recent advances in bioinformatic analysis, making it more difficult to obtain a meticulous understanding of each miRNA. Although comprehensive approaches by genome-wide profiling can address those problems to some extent, a further large-scale validation with well-established methods seems to be required in this area as well.

To overcome obstacles due to inter-individual variations, our group tried to identify candidate miRNAs by comparing miRNA profiles between pre- and post-operative samples in the same individuals^[125]. Because miRNAs are involved in various non-cancerous cell biology including physiological modulation and pathological disruption of basic pathways^[126,127], the existence of inter-individual differences can be strongly suspected based on miRNA expression. As a result, two miRNAs, miR-451 and miR-486, were selected based on this strategy and their significant value in discriminating between GC patients and healthy controls was clearly demonstrated with an AUC of 0.96 and 0.92 in ROC curve analysis for miR-451 and miR-486, respectively. We suggest that the miRNAs isolated by these concepts could be valuable biomarkers for the effective detection of early cancer and recurrence because the change of these miRNAs can be affected by the reduction of the volume of cancer tissue and is therefore directly related to tumor existence.

Most recently, our group published new observa-

tions in which long non-coding RNAs (lncRNAs) in the plasma of GC patients were successfully detected^[128]. Specifically, three lncRNAs (H19, HOX antisense intergenic RNA and metastasis associated lung adenocarcinoma transcript-1) stably exist in plasma from both GC patients and healthy controls. Plasma H19 levels were significantly higher in the patient group than the control group and decreased postoperatively, implying the possible use of H19 levels as a novel diagnostic marker in GC. lncRNAs are defined as non-protein coding transcripts longer than 200 nt lacking significant open reading frames and involved in fundamental cellular processes, such as RNA processing, gene regulation, chromatin modification, gene transcription, and post-transcriptional gene regulation based on RNA sequence complementary interactions^[129,130]. Detailed investigations have shown that lncRNAs can exhibit developmental and tissue specific expression patterns as well as aberrant regulation in a variety of diseases, including GC^[131,132]. Explorations of a novel type of RNA can provide more intriguing aspects in this research field.

CONCLUSION

Although the concept of “liquid biopsy” possesses great potential in detection and monitoring of diseases as previously described in detail, several hurdles should be

Table 5 Circulating cell-free microRNA/long non-coding RNA in gastric cancer

Characteristic and number of patients		Controls (<i>n</i>)	Plasma/serum	Detection method		Detection rate/statistic value				Ref.
microRNA I -IV	69	30	Plasma	qRT-PCR	miR-17-5p↑	<i>P</i> = 0.05	Pt. (<i>n</i> = 69) <i>vs</i> Ctrl (<i>n</i> = 30) (a)			Tsujiura <i>et al</i> ^[124]
					miR-21↑	<i>P</i> = 0.006 <i>P</i> = 0.013	Pt. (<i>n</i> = 69) <i>vs</i> Ctrl (<i>n</i> = 30) (a) paired (<i>n</i> = 10), pre-op > post-op (b)			
					miR-106a↑	<i>P</i> = 0.008	Pt. (<i>n</i> = 69) <i>vs</i> Ctrl (<i>n</i> = 30) (a)			
					miR-106b↑	<i>P</i> < 0.001 <i>P</i> = 0.022	AUC = 0.721	Pt. (<i>n</i> = 69) <i>vs</i> Ctrl (<i>n</i> = 30) (a) paired (<i>n</i> = 10), pre-op > post-op (b)		
					let-7a↓	<i>P</i> = 0.002	Pt. (<i>n</i> = 69) <i>vs</i> Ctrl (<i>n</i> = 30) (a)			
					miR-106a/let-7a↑	AUC = 0.879				
I -IV	164	127	Serum	Solexa sequencing qRT-PCR	miR-1↑	<i>P</i> < 0.01	Pt. (<i>n</i> = 164) <i>vs</i> Ctrl (<i>n</i> = 127) (a)			Liu <i>et al</i> ^[177]
					miR-20a↑	<i>P</i> < 0.01	(a)			
					miR-27a↑	<i>P</i> < 0.01	(a)			
					miR-34a↑	<i>P</i> < 0.01	(a)			
					miR-423-5p↑	<i>P</i> < 0.01	(a)			
					Five-serum miRNA signature↑	AUC = 0.879	Pt. (<i>n</i> = 22) <i>vs</i> Ctrl (<i>n</i> = 22) (test study)			
I -IV	56	30	Plasma	Microarray	miR-451↑	AUC = 0.831 <i>P</i> < 0.01	Pt. (<i>n</i> = 142) <i>vs</i> Ctrl (<i>n</i> = 105) (validation study) AUC = 0.96 Pt. (<i>n</i> = 56) <i>vs</i> Ctrl (<i>n</i> = 30) (a)			Konishi <i>et al</i> ^[125]
					qRT-PCR	<i>P</i> < 0.01	paired (<i>n</i> = 29), pre-op > post-op (b)			
					miR-486↑	<i>P</i> < 0.01 <i>P</i> < 0.01	AUC = 0.92	Pt. (<i>n</i> = 56) <i>vs</i> Ctrl (<i>n</i> = 30) (a) paired (<i>n</i> = 29), pre-op > post-op (b)		
I -IV	40	41	Serum	Microarray qRT-PCR	miR-187*↑	<i>P</i> = 0.0016	AUC = 0.704	Pt. (<i>n</i> = 40) <i>vs</i> Ctrl (<i>n</i> = 41) (a)		Liu <i>et al</i> ^[178]
					miR-371-5p↑	<i>P</i> = 0.0009	AUC = 0.715	(a)		
N/A	82	82	Serum	Microarray	miR-378↑	<i>P</i> < 0.0001	AUC = 0.861	(a)		Song <i>et al</i> ^[179]
					miR-221↑	AUC = 0.74	Pt. (<i>n</i> = 68) <i>vs</i> Ctrl (<i>n</i> = 68) (second validation study)			
N/A (pre-op, post-op and recurrent)	20		Serum	qRT-PCR	miR-376c↑	AUC = 0.71	Pre-op (<i>n</i> = 20) > post-op (<i>n</i> = 20) (a)			Tsai <i>et al</i> ^[180]
					miR-744↑	AUC = 0.71	Post-op (<i>n</i> = 20) < at recurrent (<i>n</i> = 20) (a)			
I -IV	30	39	Serum	qRT-PCR	miR-196a	<i>P</i> = 0.012 <i>P</i> = 0.002	(a)			Wang <i>et al</i> ^[181]
I -IV	87		Plasma	qRT-PCR	miR-21↑	<i>P</i> < 0.001	AUC = 0.81	Pt. (<i>n</i> = 30) <i>vs</i> Ctrl (<i>n</i> = 39) (a)		
	(65 pre-op)				miR-17-5p↑	<i>P</i> < 0.001	Pre-op (<i>n</i> = 65) > post-op (<i>n</i> = 16) (a)			Wang <i>et al</i> ^[182]
						<i>P</i> = 0.003	Post-op (<i>n</i> = 16) < recurrent (<i>n</i> = 6) (a)			
	(16 post-op)					OS: <i>P</i> = 0.0003	miR-17-5p high <i>vs</i> low	Pre-op (<i>n</i> = 65) (c)		
					miR-20a↑	<i>P</i> = 0.006	Pre-op (<i>n</i> = 65) > post-op (<i>n</i> = 16) (a)			
						OS: <i>P</i> = 0.0003	miR-20a high <i>vs</i> low	Pre-op (<i>n</i> = 65) (c)		
	(6 recurrent)					OS: <i>P</i> = 0.013	miR-20a high <i>vs</i> low	pre-op (<i>n</i> = 65) (d)		
I -IV	20	20	Serum	miRNA microarray qRT-PCR	miR-375↓	<i>P</i> < 0.001	AUC = 0.835	Pt. (<i>n</i> = 20) <i>vs</i> Ctrl (<i>n</i> = 20) (a)		Zhang <i>et al</i> ^[183]
	20	190	Plasma	miRNA microarray	miR-195-5p↓	<i>P</i> < 0.05	Fold changes 13.3	Pt. (<i>n</i> = 20) <i>vs</i> Ctrl (<i>n</i> = 130) (a)		Gorur <i>et al</i> ^[184]
I -III (25 without LN meta) (54 with LN meta)	79				miR-21↑	<i>P</i> < 0.001	Correlation with pN stage (e)			Kim <i>et al</i> ^[185]
					miR-146a↑	<i>P</i> = 0.001	Correlation with pN stage (e)			
					miR-148a↑	<i>P</i> < 0.001	Correlation with pN stage (e)			
I -IV	69		Plasma	qRT-PCR	miR-21↑	CSS: <i>P</i> = 0.0451	miR-21 high <i>vs</i> low (c)			Komatsu <i>et al</i> ^[186]

I - II	80	70 (healthy controls) 20 (precancerous disease)	Plasma	qRT-PCR	miRNA-199a-3p↑	CSS: $P = 0.0133$	(d)			
						$P < 0.001$	AUC = 0.818	Pt. ($n = 80$) vs Ctrl. ($n = 70$)	(a)	Li <i>et al</i> ^[187]
						$P = 0.004$	Pt. ($n = 80$) vs pancreatic disease ($n = 20$)		(a)	
						$P = 0.012$	Pre-op > post-op ($n = 30$)		(b)	
Long non-coding RNA										
I -IV	43	33	Plasma	qRT-PCR	H19	$P = 0.029$		Pt. ($n = 43$) vs Ctrl. ($n = 33$)	(a)	Arita <i>et al</i> ^[128]
					HOTAIR	$P = 0.096$			(a)	
					MALAT1	$P = 0.14$			(a)	

a: Unpaired *t*-test/Mann-Whitney *U*-test; b: Wilcoxon test; c: Kaplan-Meier survival curves, Log-rank test; d: Logistic regression model (multivariate); e: Kruskal-Wallis test. AUC: Area under the receiver operating characteristic curve; CSS: Cause-specific survival; LN: Lymph node; N/A: Not available; qRT-PCR: Quantitative real-time polymerase chain reaction; OS: Overall survival.

overcome before translating it into clinical settings. One of the most important issues is the lack of consensus in technical approaches, which involves various aspects of the methodologies, such as preferable sample type, storage conditions, candidate molecules and suitable detection techniques. Moreover, technical errors may introduce contaminated cells or molecules into experimental samples, which could cause misunderstandings and statistical errors. Therefore, the standardization of techniques throughout all experimental steps should be emphasized.

Owing to recent remarkable technological developments, novel revolutionary approaches including an *in vivo* CTC isolation system^[32] and multi-detectable array have been introduced into this research field. However, some issues raised by those advances should be addressed properly. Although multi-detection approaches can facilitate exhaustive screenings and provide us with various types of information, the important considerations are which molecules should be selected as a tumor marker and how the result of an individual patient obtained by multiple detection panels should be effectively utilized. Of course, the cost and practicality of each assay should also be taken into consideration to some extent.

In summary, the science of CTCs and circulating cfNAs remains in its infancy. Despite numerous approaches and techniques that have been advocated to accomplish the ultimate goal, that is, the development of a useful, sensitive and real-time monitoring system from the blood, few proposals have been translated into clinical practice. Large-scale studies and further understanding of their biology and significance could resolve those problems and enhance their utility as biomarkers. Consequently, the development of novel biomarkers based on CTCs and cfNAs could provide many benefits for cancer patients including the improvement of clinical outcomes in the near future.

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Cortactin expression confers a more malignant phenotype to gastric cancer SGC-7901 cells

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Abstract

AIM: To study the effects of cortactin on the tumor biology of SGC-7901 cells and identify the mechanism involved in the process.

METHODS: Cell lines in which cortactin was stably overexpressed or knocked down as well as the respective control cell lines were established by standard molecular methods. The effects of cortactin on the proliferation, migration and invasion capacity of SGC-7901 cells were assessed by the MTT assay, colony formation, flow cytometry, transwell migration and matrigel invasion. Nude mouse models were also used to assess the role of cortactin in the growth and metastasis of SGC-7901 cells *in vivo*. Western blotting analysis was performed to detect the expression of epidermal growth factor receptor (EGFR) and downstream molecules.

RESULTS: Cell lines in which cortactin was stably overexpressed or knocked down as well as control cell

lines were successfully established and designated as LV5-cortactin-SGC, LV5-SGC, LV3-shRNA-SGC and LV3-SGC. Cortactin overexpression promoted SGC-7901 cell migration (340.7 ± 12.6 vs 229.1 ± 23.2 , $P < 0.01$) and invasion (71.6 ± 5.2 vs 48.4 ± 3.6 , $P < 0.01$). Cortactin downregulation impaired SGC-7901 cell migration (136.2 ± 19.8 vs 225 ± 17) and invasion (29.2 ± 5.2 vs 49.6 ± 3.8 , $P < 0.01$). The results from the MTT and colony formation assays results indicated increased LV5-cortactin-SGC cell proliferation and decreased LV3-shRNA-SGC cell proliferation compared to the control cells. Flow cytometry analysis demonstrated that cortactin overexpression promoted the proliferation index of SGC-7901 cells, and the results were reversed when cortactin was downregulated. Mouse tumor models confirmed that cortactin expression increased SGC-7901 cell proliferation and metastasis *in vivo*. Western blotting analysis revealed that cortactin elevated EGFR expression and activated the downstream molecules.

CONCLUSION: Cortactin expression promoted the migration, invasion and proliferation of SGC-7901 cells both *in vivo* and *in vitro*. The EGFR signaling pathway is mechanistically involved.

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Key words: Gastric cancer; Cortactin; Epidermal growth factor receptor; Invasion; Metastasis; Proliferation

Core tip: Cortactin is an actin-related protein 2/3 complex-activating and filamentous (F)-actin-binding protein that is implicated in tumor cell motility and metastasis. Clinical studies have shown that cortactin overexpression is often associated with the clinicopathological parameters and poor prognosis in a variety of cancers, including gastric cancer. In this study, the effects of cortactin on gastric cancer progression were investigated. The results showed that cortactin expression promoted SGC-7901 cell migration, invasion and

proliferation both *in vitro* and *in vivo*. The epidermal growth factor receptor signaling pathway is mechanistically involved. Cortactin may serve as a novel therapeutic target of gastric cancer.

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INTRODUCTION

Gastric carcinoma is one of the most common cancers in the world and the second leading cause of cancer death worldwide^[1-3]. Over 70% of new cases and deaths occur in developing countries and the highest incidence rate is in China and other eastern Asian countries^[3]. One recent study indicated that the 5-year overall survival of the disease was 53%^[4]. In contrast, the 5-year survival rates for all tumor stages are only 20% to 25% in the western world, and the median survival time approximately 24 mo^[5]. Surgery and adjuvant chemo-radiotherapy are traditional therapies that have a poor prognosis, therefore molecular targeted therapy has been studied extensively. Targeted therapy is based on a comprehensive understanding of the mechanisms governing gastric cancer progression, but an exact mechanism has yet to be elucidated.

Cortactin protein was first identified in chicken cells transformed by the src oncogene^[6]. The gene encoding cortactin maps to chromosome 11q13, which is often amplified in many carcinomas, such as head and neck squamous cell carcinoma (HNSCC)^[7]. Cortactin is an actin-related protein 2/3 (Arp2/3) complex-activating and filamentous (F)-actin-binding protein that is implicated in tumor cell motility and metastasis^[8]. The protein is enriched in cortical structures such as membrane ruffles and lamellipodia^[9]. The properties of cortactin indicate that it may be important for microfilament-membrane interactions as well as the transduction of signals from the cell surface to the cytoskeleton^[9]. In carcinoma cells that constitutively overexpress cortactin, this protein accumulates in the cytoplasm as well as protruding leading lamellae or podosome-like structures, thereby contributing to the invasive potential of these tumor cells^[10]. Cortactin overexpression often occurs in malignant tumors regardless of the chromosome 11q13 amplification and is associated with poor prognosis^[11-15]. Cortactin has also been studied in gastric carcinoma tissues. The results indicated that cortactin overexpression directly correlates with more advanced cancer and lymph node stages as well as degree of differentiation; thus, cortactin overexpression is associated with unfavorable survival^[16,17]. However, the exact role of cortactin in gastric cancer progression remains unknown.

In this study, molecular methods were used to overexpress and knockdown cortactin in SGC-7901 gastric carcinoma cells and the resulting phenotypes were analyzed. The overexpression of cortactin promoted the migration, invasion and proliferation of SGC-7901 cells *in vitro*. Cortactin overexpression enhanced tumor growth and metastatic capacity *in vivo*. The Western blotting results indicated that epidermal growth factor receptor (EGFR) expression was upregulated in the cortactin overexpression cells and that the downstream molecules were activated. Cortactin silencing resulted in the opposite effects. These observations are consistent with a previous report indicating that cortactin overexpression in a cervical cancer cell line markedly inhibits the ligand-induced down-regulation of EGFR^[18]. In the same study, the RNAi-mediated reduction of cortactin expression in an 11q13-amplified HNSCC cell line accelerated EGFR degradation^[18]. Therefore, cortactin and EGFR may synergistically contribute to the progression of gastric carcinoma. Cortactin may serve as a promising therapeutic target in gastric cancer.

MATERIALS AND METHODS

Cell culture

SGC-7901 cells (obtained from Professor Liang, School of Medicine, Tongji University) were cultured in RPMI 1640 (Gibco, Grand Island, NY, United States) supplemented with 10% fetal calf serum (FCS) and maintained at 37 °C in 5% CO₂. HEK293T cells were cultured in DMEM with high glucose supplemented with 10% FCS and maintained at 37 °C in 5% CO₂.

Antibodies and plasmids

Rabbit polyclonal anti-cortactin, anti-phospho-cortactin, anti-EGFR, anti-AKT, anti-phospho-AKT, anti-signal transducer and activator of transcription 3 (STAT3), anti-phospho-STAT3, anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and the secondary mouse anti-rabbit antibody were purchased from Epitomics (Burlingame, CA, United States). For overexpression and knockdown of cortactin, the pGLV5/H1/GFP+Puro and pGLV3/H1/GFP+Puro lentiviral plasmid were used, respectively. The PG-P1-VSVG, PG-P2-REV and PG-P3-RRE helper plasmids were employed to package the lentiviral vector (Genepharma, Shanghai, China).

The design of shRNA to silence the expression of cortactin

Two following validated cortactin shRNA sequences were used, (forward): 5'-GATCCGAAAGAC-TACTCCAGTGGTTTCAAGAGAACCCTG-GAGTAGTCTTTCTTTTTCG-3', and (reverse): 5'-AATTCAAAAAAGAAAGACTAC TCCAGTG-GTTCTCTTGAAACCCTGGAGTAGTCTTTTCG-3'. The non-specific control sequences were as follows: (forward) 5'-GATCCGTTCTC CGAACGTGTCAC-GTTTCAAGAGAACGTGACACGTTCCGGAGAAGT

TTTT TG-3', and (reverse) 5'-AATTCAAAAACCTA-AGGTTAAGTCGCCCTCGCT CGAG CGAGGGC-GACTTAACCTTAGG-3'. The two pairs were annealed and inserted into the *Bam*HI and *Eco*RI sites of the pGLV3/H1/GFP+Puro lentiviral plasmid. The product was then transformed into Trans5 α (TransGen Biotech, Beijing, China). Positive clones were selected and verified by restriction enzyme and sequence analysis.

Construction of cortactin overexpression plasmid

The cortactin coding sequence (CDS) was obtained by polymerase chain reaction (PCR) using the TransTaq High Fidelity PCR SuperMix I (TransGen Biotech, Beijing, China) in accordance with the manufacturer's protocol. The following primers were used: (forward) 5'-ATAAGAATGCGGCCGCATG TG-GAAAGCTTCAGCAGGCCACG-3', and (reverse) 5'-CGGGATCCCTACT GCCGCAGCTCCA-CATAGTTG-3'. The cortactin CDS was inserted into to the *No*I and *Bam*HI sites of the pGLV5/H1/GFP+Puro lentiviral plasmid, and the product was transformed into Trans5 α (TransGen Biotech, Beijing, China). Positive clones were selected and verified by PCR and sequence analysis.

Packaging of lentiviral vectors and the establishment of stable transfectants

The successfully constructed pGLV3-cortactin shRNA and the pGLV5-cortactin vector were transfected into HEK293T cells with PG-P1-VSVG, PG-P2-REV and PG-P3-RRE plasmids. All transfection reactions were performed using the GeneExpresso Max Transfection Reagent (Excellgen; Rockville, MD, United States) in accordance with the manufacturer's instructions. Eight to twelve hours after transfection, the culture medium of the HEK293T cells was changed to fresh culture medium. The supernatant was then harvested at 72 h and titered using the HEK293T cell line. The pGLV3-control shRNA and pGLV5-control plasmids were also packaged simultaneously. The lentiviral stock titer was 1×10^8 TU/mL. SGC-7901 cells were cultured in six-well tissue culture plates and infected with the four lentiviral vectors at a multiplicity of infection of 40 for 24 h. The medium was replaced with fresh complete medium. After 3 d, cells were observed by fluorescence microscopy to confirm that greater than 90% of the cells were GFP-positive (Figure 1). Subsequently, the GFP-positive cells were screened by addition of 5 μ g/mL puromycin. The resultant stable cell lines were designated as LV5-cortactin-SGC, LV5-SGC, LV3-shRNA-SGC, and LV3-SGC.

Real-time quantitative reverse transcription PCR

Real time reverse transcription (RT) polymerase chain reactions were performed as previously described using GAPDH as an internal control^[19]. The primer sequences used for q-RT-PCR are as follows: (cortactin forward primer) 5'-TGAGTGTGTGTTCTTCCCCAAG-3', (cortactin reverse primer) 5'-CACGTGACCTTCTG-

GAAAGACA-3'. (GAPDH forward primer) was 5'-GTGGTCTCCTCTGACTTCAACA-3', (GAPDH reverse primer): 5'-GTTGCTG TAGCCAAATTCGTTGT-3'. All the experiments were performed in triplicate with 3 replicates.

Western blotting assay

Western blotting was performed as previously described^[19] with antibodies against cortactin, phosphor-cortactin, GAPDH, EGFR, STAT3, phosphor-STAT3, AKT and phosphor-AKT at 1:1000 dilutions. The band intensity was detected using Image Quant LAS 4000 (GE Healthcare, Buckinghamshire, England) and analyzed using ImageJ software.

Cell migration and invasion assays

Cell migration and invasion assays were performed using 24-well Transwell chambers with a pore size of 8 μ m (Costar, New York, NY, United States) planted with 5×10^4 cells in serum-free RPMI 1640. The lower chambers were filled with medium that contained 10% fetal bovine serum as the chemoattractant. For the invasion assays, the inserts were coated with 50 μ L Matrigel (1:3 dilution; BD Bioscience, Franklin Lakes, NJ, United States). After culture for 24 h, the cells on the upper membrane surface were removed by scraping with a cotton swab, and the cells that passed through the filter were fixed and stained using the hematoxylin-eosin reagent. The invading cells were counted in five randomly selected high power fields using a microscope. All the experiments were performed in triplicate with 3 replicates and the mean was calculated.

Cell proliferation assay

In general, 1×10^3 cells were seeded per well in a 96-well culture plate. Fifty microliters of 5 mg/mL 3-(4,5)-dimethylthiazazo(-z-y1)-3,5-diphenyltetrazolium-bromide (Kengen, Nanjing, China) in $1 \times$ phosphate-buffered saline was added to growth medium 48 and 72 h after plating. The cells were incubated for 4 h at 37 $^{\circ}$ C and then solubilized with 150 μ L DMSO for 30 min. A 96-well plate reader (Bio-Rad, Philadelphia, United States) was used to measure the absorbance at 490 nm. All the experiments were performed in triplicate with 3 replicates and the mean was calculated.

Colony formation assay

After achieving 70% to 80% confluence, the cells were trypsinized, washed three times with phosphate-buffered saline (PBS), and then plated in triplicate at 1×10^3 cells/6 cm well. After 14 d of culture, the colonies were fixed with 4% paraformaldehyde and stained with crystal violet. The number of colonies per well was counted using a microscope and a cluster of 50 cells was designated as a colony. Three independent experiments were performed.

Flow cytometry analysis

Cells were plated at 2×10^5 cells per well in a 6-well cul-

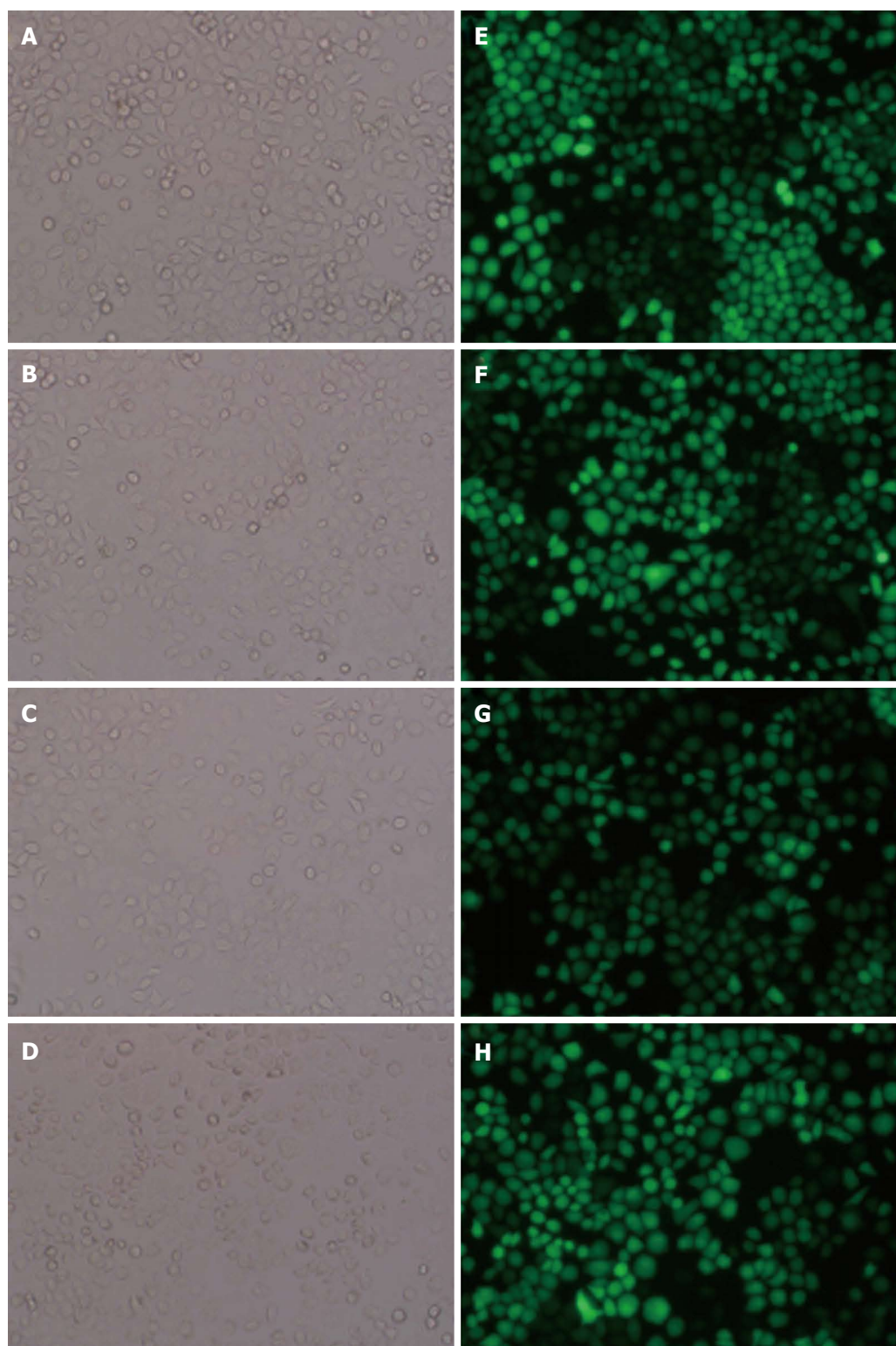


Figure 1 Establishment of the LV5-cortactin-SGC, LV5-SGC, LV3- shRNA-SGC, and LV3-SGC stable cell lines. A, E: LV5-cortactin-SGC; B, F: LV5-SGC; C, G: LV3-shRNA-SGC; D, H: LV3-SGC. The stable transfectants expressed green fluorescent protein. The left half of the figure was obtained under light field, and the right half was obtained under fluorescence. There is no morphological difference found between the stable cell lines.

ture plate and cultured for 48 h. The cells were then harvested, fixed with 70% ethanol, and washed with ice-cold PBS. The cells were stained with 50 $\mu\text{g/mL}$ propidium iodide and 250 $\mu\text{g/mL}$ RNase and incubated for 30 min in the dark at room temperature. The cells were subsequently analyzed by flow cytometry using a FACS Calibur

(Becton Dickinson, Bedford, MA, United States).

In vivo assays of tumor growth and metastasis

Four- to six-week-old female BALB/c nude mice were purchased from the Slac Laboratory Animal Company (Shanghai, China) and fed in the Experimental Animal

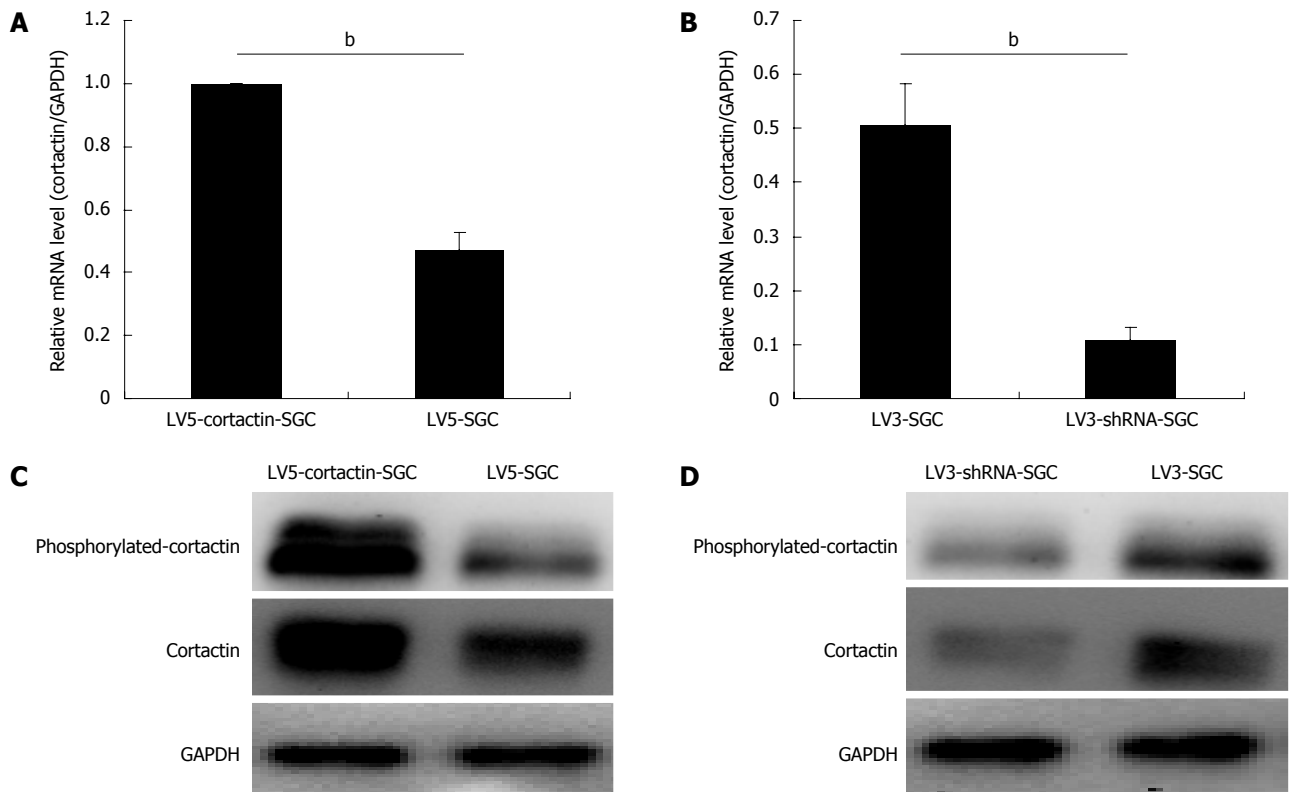


Figure 2 Cortactin expression at the mRNA and protein level in the recombinant cell lines. A, B: Bar chart represents quantitative real-time polymerase chain reaction assessment of the cortactin mRNA levels in the LV5-cortactin-SGC, LV5-SGC, LV3-SGC and LV3-shRNA-SGC cells ($P < 0.01$ between groups, Student's *t*-test); C: Western blotting analysis of cortactin and phosphorylated cortactin in the LV5-cortactin-SGC and LV5-SGC control cells. The cortactin and phosphorylated cortactin increased greatly in the LV5-cortactin-SGC cells compared with the LV5-SGC cells; D: Western blotting analysis of cortactin and phosphorylated cortactin in the LV3-shRNA-SGC and LV3-SGC control cells. The levels of cortactin and phosphorylated cortactin were decreased significantly in the LV3-shRNA-SGC cells compared with LV3-SGC cells. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control.

Center of Tongji University. All mice were maintained in a germ-free environment with free access to food and water. To examine the effects of cortactin on tumor cell proliferation and metastasis *in vivo*, LV5-cortactin-SGC, LV5-SGC, LV3-shRNA-SGC and LV3-SGC cells were used. For the *in vivo* proliferation assay, four nude mice from each group were injected subcutaneously with 5.0×10^6 cells. The tumor volumes were measured at the indicated times according to the following formula^[20]: $0.5 \times \text{length} \times \text{width}^2$. After 8 wk, the mice that were injected subcutaneously were sacrificed and the tumors were dissected and measured to calculate the volume. For the spontaneous metastasis assay, the cells were injected into the inferior portion of the gastric serosa of 8 nude mice in each group. The mice were monitored every 3 d and sacrificed 12 wk after injection. The livers of the mice were dissected, and the liver metastases were evaluated. Paraffin-embedded tumors and livers were sectioned, and stained with hematoxylin and eosin. The slides were microscopically observed to confirm the presence of the tumor formation and metastasis in the mice. The average values were expressed as the mean \pm SE. This animal experiment was approved by the ethic commission of the experimental center of Tongji University. The study was in accordance with the recommendations of the regional and country animal ethics committee.

Statistical analysis

The results are expressed as the mean \pm SD. For the comparison of the means between two groups, a two-tailed *t*-test was used. For the statistical analysis of the *in vivo* metastases, the Mann Whitney *U* test was used. Statistical analysis was performed using SPSS software version 17.0. A *P*-value < 0.05 was considered statistically significant.

RESULTS

The establishment and identification of stable transfectants

Four stable recombinant cell lines were generated using the pGLV5-cortactin, pGLV5-control, pGLV3-cortactin shRNA and pGLV3-control lentiviruses, which were designated as LV5-cortactin-SGC, LV5-SGC, LV3-shRNA-SGC and LV3-SGC cells, respectively. As shown in Figure 1, the stable transfectants expressed green fluorescent protein, and no morphological differences were observed between the stable cell lines. Quantitative real-time PCR was performed to identify the levels of cortactin gene transcription in the cell lines. Cortactin mRNA transcription was significantly increased in LV5-cortactin-SGC cells compared with the LV5-SGC cells ($P < 0.01$, Figure 2A) and significantly reduced in the LV3-shRNA-SGC

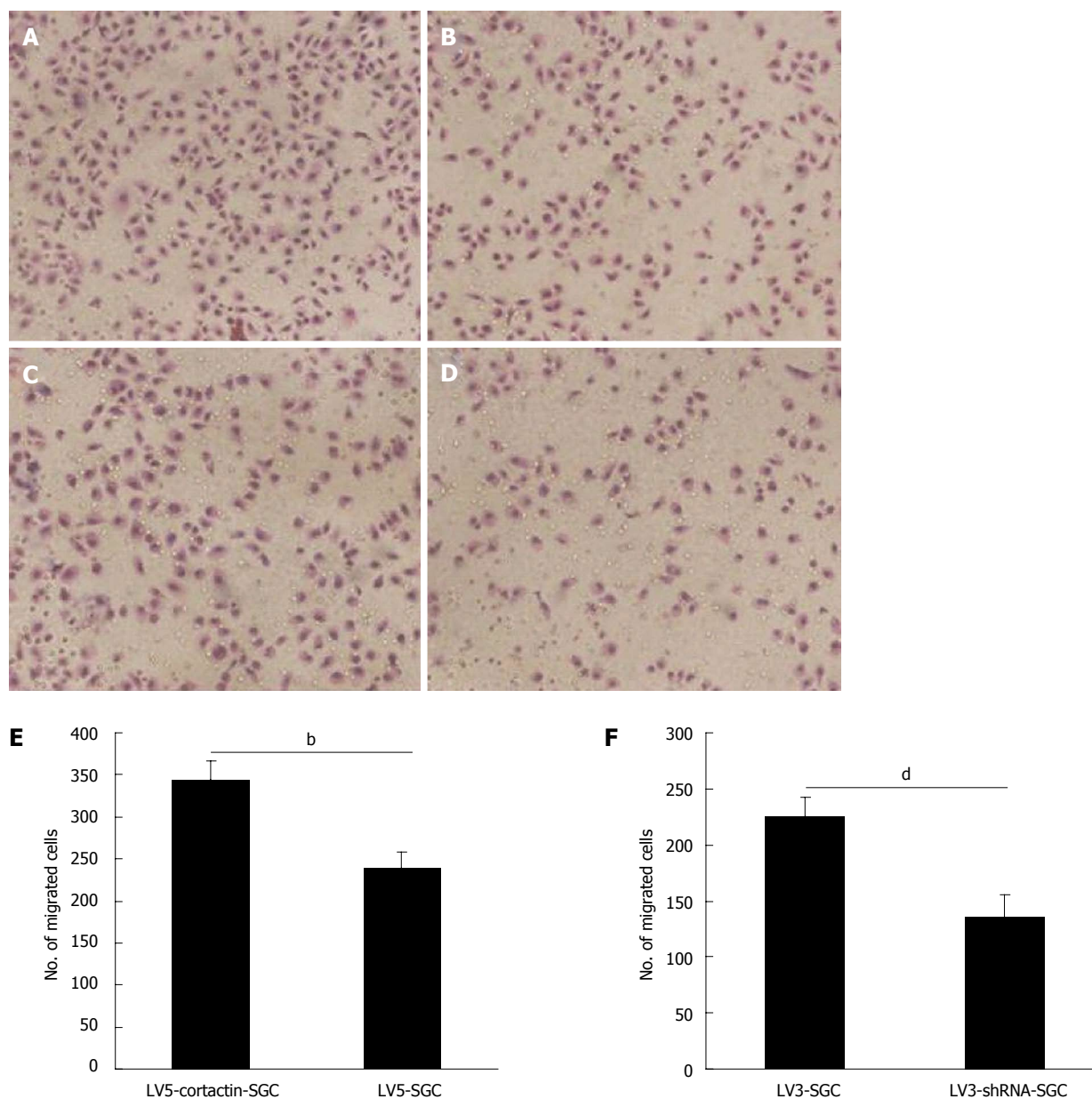


Figure 3 Cortactin expression promotes SGC-7901 cell migration. A-D: Number of LV5-cortactin-SGC cells (A) that migrated through the chamber was greatly increased compared with that for the LV5-SGC cells (B). The number of LV3-shRNA-SGC cells (D) that migrated through the chamber was greatly decreased compared with the number of migrating LV3-SGC cells (C); E: Statistical analysis of invasion by LV5-cortactin-SGC and control LV5-SGC cells (340.7 ± 12.6 vs 229.1 ± 23.2 , $^bP < 0.01$); F: Statistical analysis of invasion by LV3-shRNA-SGC and control LV3-SGC cells (136.2 ± 19.8 vs 225 ± 17 , $^dP < 0.01$). Student's *t*-test was used, and $P < 0.05$ was considered statistically significant.

cells compared with the LV3-SGC cells ($P < 0.01$, Figure 2B). Western blotting analysis showed that the cortactin protein expression was increased by 2.6-fold in the LV5-cortactin-SGC cells compared with the LV5-SGC cells (Figure 2C). Cortactin protein expression was decreased by 70% in the LV3-shRNA-SGC cells compared with the LV3-SGC cells (Figure 2D). These results suggested that the inserted genes were highly efficient in the stable transfectants.

Cortactin promote the SGC-7901 cell migration and invasion in vitro

To determine the role of cortactin expression in gastric cancer cell migration and invasion, Transwell migration

and invasion assays were performed. For the migration assay, Transwell chambers were used in absence of Matrigel. LV5-cortactin-SGC cell migration was significantly enhanced compared with that of LV5-SGC cells. The number of LV5-cortactin-SGC cells that migrated to the lower chamber was 340.7 ± 12.6 per high-power field (HPF) compared with 229.1 ± 23.2 per HPF for the LV5-SGC cells ($P < 0.01$; Figure 3A, B and E). Simultaneously, LV3-shRNA-SGC cell migration greatly decreased compared with that of LV3-SGC cells. The mean number of LV3-shRNA-SGC cells that migrated to the lower chamber was 136.2 ± 19.8 per HPF compared with 225 ± 17 per HPF for the LV3-SGC cells ($P < 0.01$; Figure 3C, D and F). For the invasion assay, Matrigel, an artificial

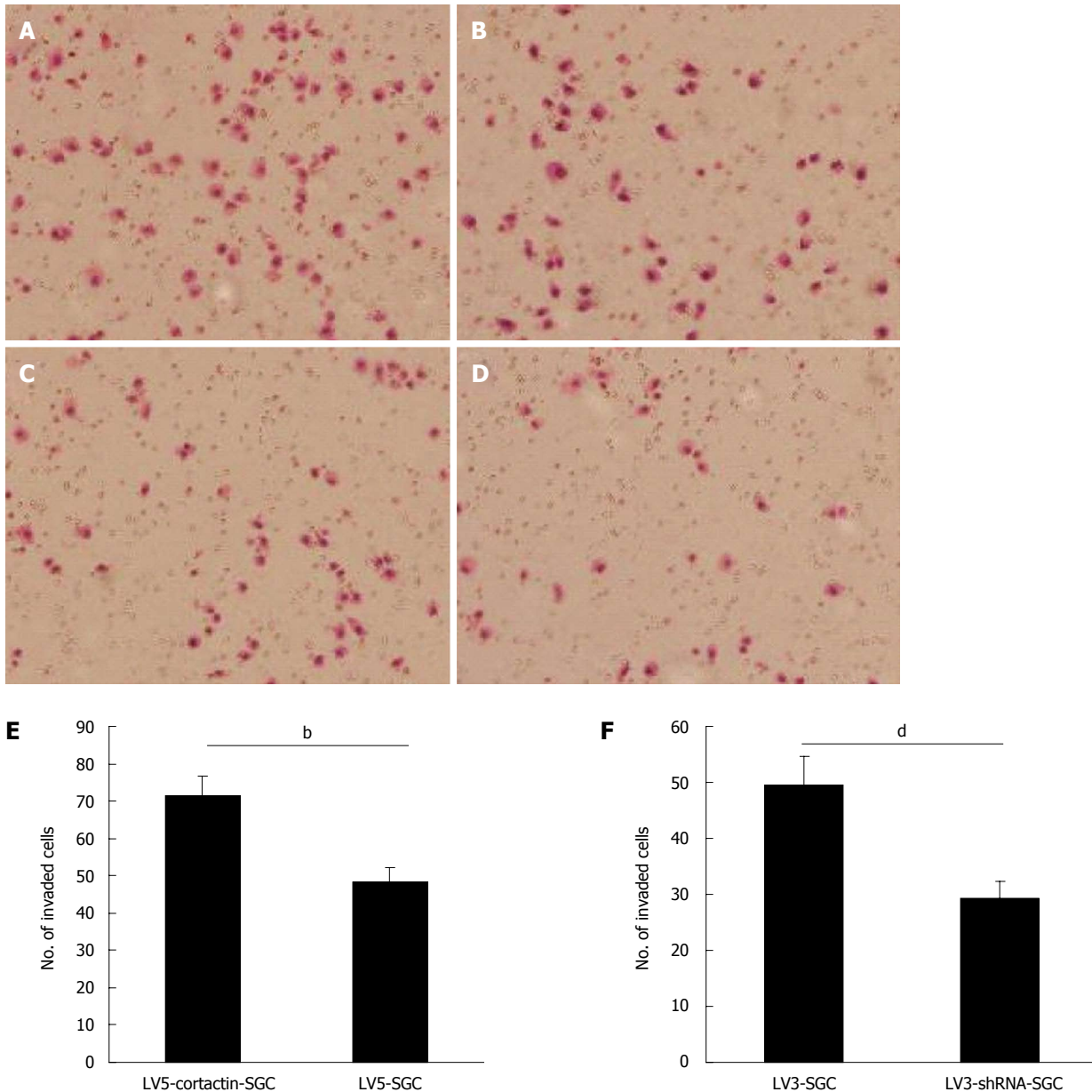


Figure 4 Cortactin expression promotes SGC-7901 cell invasions. A-D: Number of LV5-cortactin-SGC cells (A) that invaded the chamber was greatly increased compared with the number of invading LV5-SGC cells (B). The number of LV3-shRNA-SGC cells (D) that invaded the chamber was greatly decreased compared with the number of invading LV3-SGC cells (C); E: Statistical analysis of invasion by LV5-cortactin-SGC and control LV5-SGC cells (71.6 ± 5.2 vs 48.4 ± 3.6 , $^bP < 0.01$); F: Statistical analysis of invasion by LV3-shRNA-SGC and control LV3-SGC cells (49.6 ± 3.8 vs 29.2 ± 5.2 , $^dP < 0.01$). Student's *t*-test was used, and $P < 0.05$ was considered statistically significant.

extracellular matrix, was used. Similarly, LV5-cortactin-SGC cell invasion was significantly enhanced compared with that of the LV5-SGC cells (71.6 ± 5.2 vs 48.4 ± 3.6 , $P < 0.01$; Figure 4A, B and E). Compared with LV3-SGC cells, the LV3-shRNA-SGC cells displayed reduced invasion (49.6 ± 3.8 vs 29.2 ± 5.2 , $P < 0.01$; Figure 4C, D and F). These results indicated that cortactin expression promotes the migration and invasion potential of SGC-7901 gastric cancer cells.

Cortactin promotes SGC-7901 gastric cancer cell proliferation

To investigate the effect of cortactin expression on gas-

tric cancer cell growth, the MTT and colony formation assays were performed. The MTT assay results indicated an obvious increase in LV5-cortactin-SGC cell proliferation compared with the LV5-SGC cells on the second and the third days ($P < 0.05$; Figure 5A). The proliferation capacity of the LV3-shRNA-SGC cells significantly decreased compared with the LV3-SGC cells on the second ($P < 0.05$; Figure 5B) and the third days ($P < 0.01$; Figure 5B). The effect of cortactin on SGC-7901 cell growth was also confirmed by the colony formation assay. These results indicated that cortactin overexpression clearly contributes to an increased ability to form colonies ($P < 0.01$; Figure 5C, D and G). Moreover, cortactin

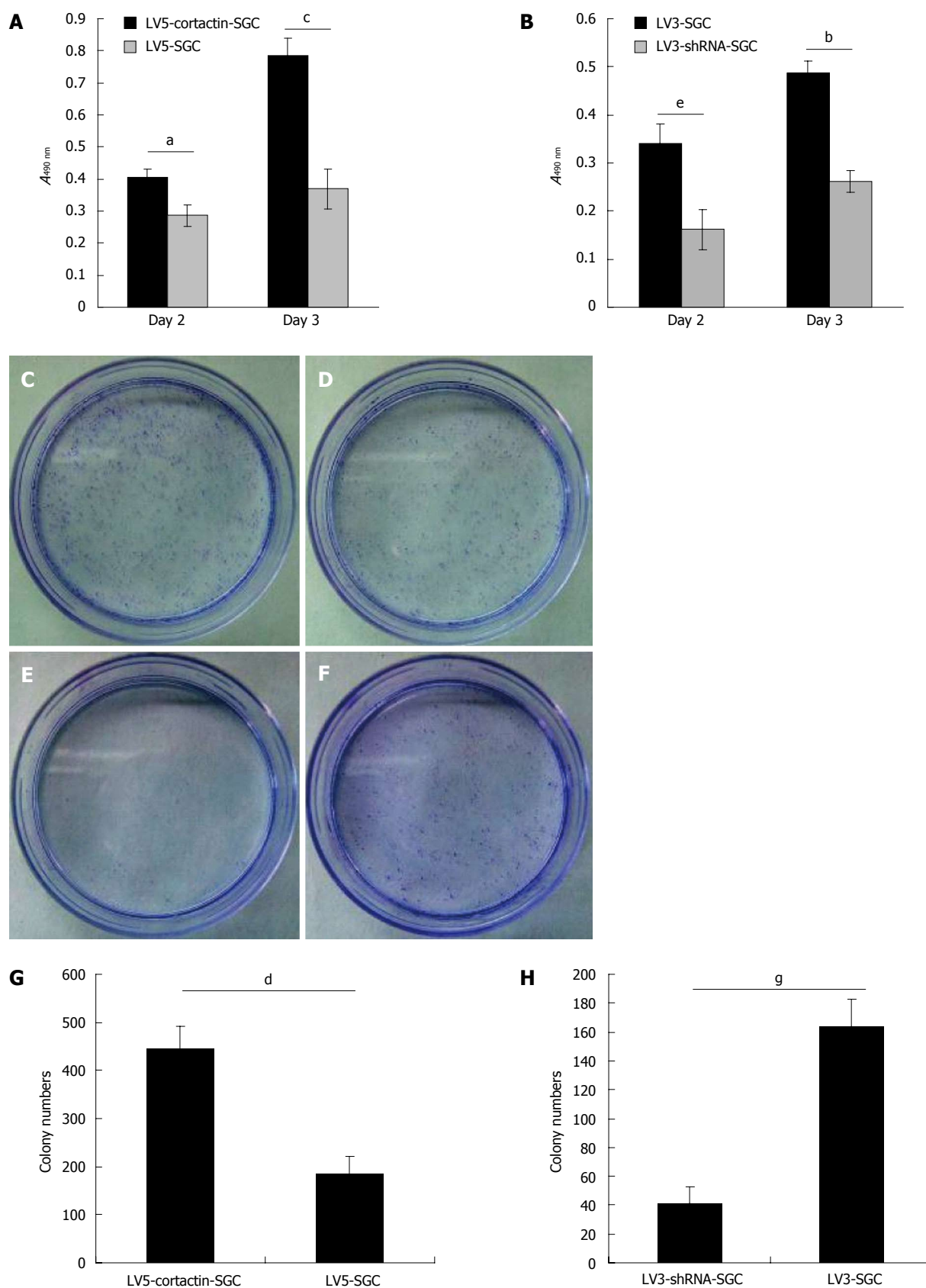


Figure 5 Cortactin expression promotes SGC-7901 cell proliferation. A: MTT assay results showed that the LV5-cortactin-SGC cell proliferation was increased on the second ($^aP < 0.05$ vs LV5-SGC cells) and the third ($^cP < 0.05$ vs LV5-SGC cells) days after plating; B: LV3-shRNA-SGC cell proliferation was significantly lower on the second ($^eP < 0.05$ vs LV3-SGC cells) and third ($^bP < 0.01$ vs LV3-SGC cells) days after plating. The colony formation assay confirmed the role of cortactin in SGC-7901 cell proliferation. The LV5-cortactin-SGC, LV5-SGC, LV3-shRNA-SGC and LV3-SGC cell colonies were stained with crystal violet (C-F, respectively); G: The number of LV5-cortactin-SGC cell colonies was greater than LV3-SGC cell colonies ($^dP < 0.01$ vs the number of LV5-SGC cell colonies); H: The number of LV3-shRNA-SGC cell colonies was significantly less than of LV3-SGC cell colonies ($^gP < 0.05$ vs the number of LV5-SGC cell colonies). Student's *t*-test was used, and $P < 0.05$ was considered significantly significant.

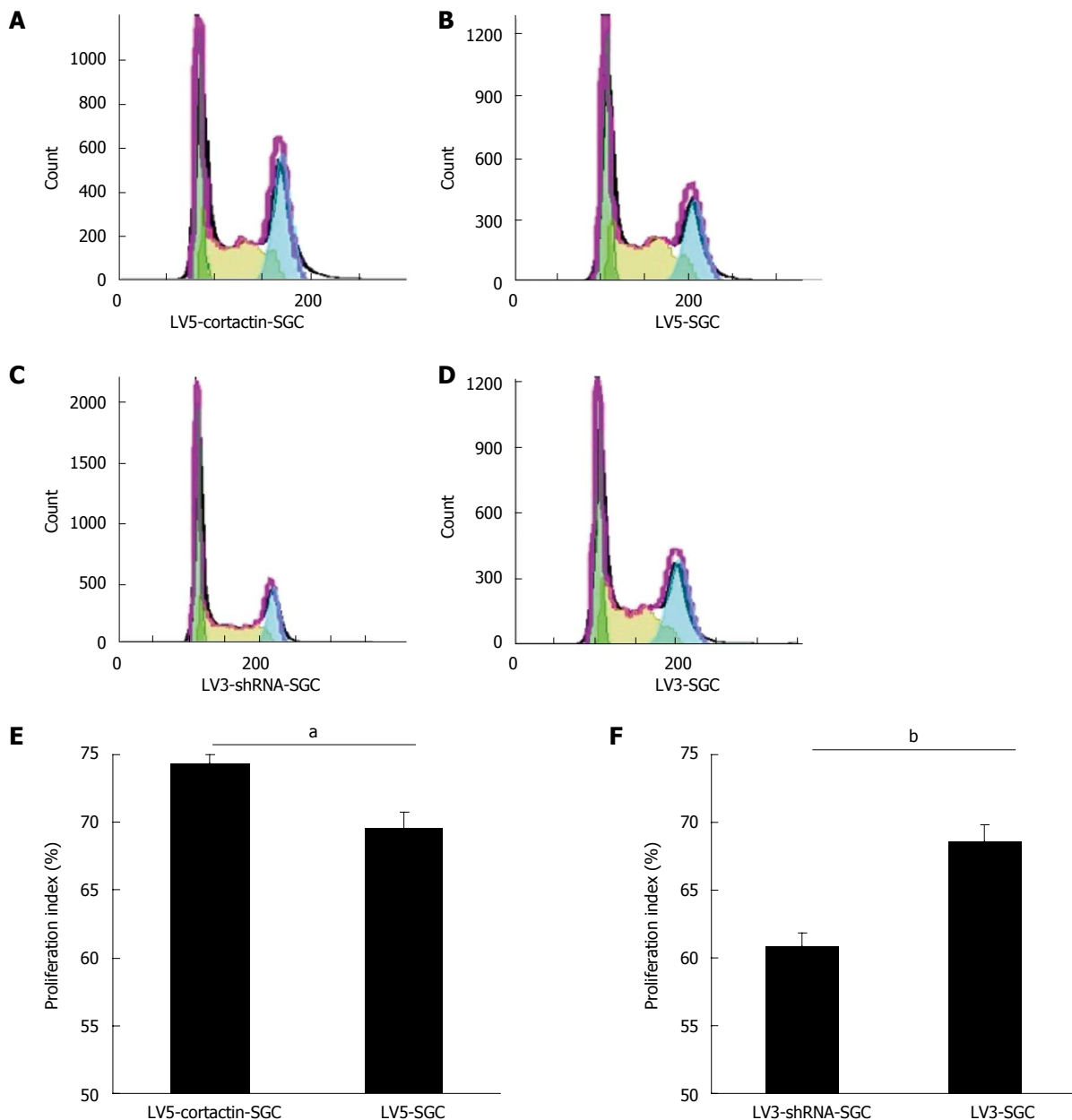


Figure 6 Effect of cortactin on the cell cycle as analyzed by flow cytometry. A-D: Cell-cycle stages of LV5-cortactin-SGC, LV5-SGC, LV3-shRNA-SGC and LV3-SGC cells were detected by flow cytometry; E: Proliferation indexes of LV5-cortactin-SGC and LV5-SGC cells, ^a $P < 0.05$ between groups was assessed by Student's *t*-test; F: Proliferation indexes of LV3-shRNA-SGC and LV3-SGC cells, ^b $P < 0.01$ between groups was assessed by Student's *t*-test. $P < 0.05$ was considered to be significantly significant.

downregulation significantly reduced of the colony formation capacity of the SGC-7901 cells ($P < 0.05$; Figure 5E, F and H). These results suggest that cortactin expression influences SGC-7901 cell growth and proliferation.

Cortactin increases the proliferation index of SGC-7901 cells

To investigate the effects of cortactin expression on the cell cycle in SGC-7901 cells, the flow cytometry assay was performed. The proliferation index was calculated as $(S + G_2M)/(G_0G_1 + S + G_2M)$ according to a previous study^[21]. The LV5-cortactin-SGC cell proliferation index was significantly higher than that of LV5-SGC cells ($P < 0.05$; Figure 6A, B and E). The proliferation index of the LV3-

shRNA-SGC cells was significantly reduced compared with that of LV3-SGC cells ($P < 0.01$; Figure 6C, D and F). These results suggest that cortactin increases the proliferation index of SGC-7901 cells.

Cortactin overexpression accelerates tumor growth and metastasis *in vivo*

To investigate the role of cortactin overexpression in the tumor growth and metastasis *in vivo*, the tumor formation and metastatic abilities of the LV5-cortactin-SGC, LV5-SGC, LV3-shRNA-SGC and LV3-SGC cells were compared in a mouse tumor model. For the tumor growth assay, the cells were inoculated subcutaneously in the nude mice and the tumor growth was measured weekly for 2

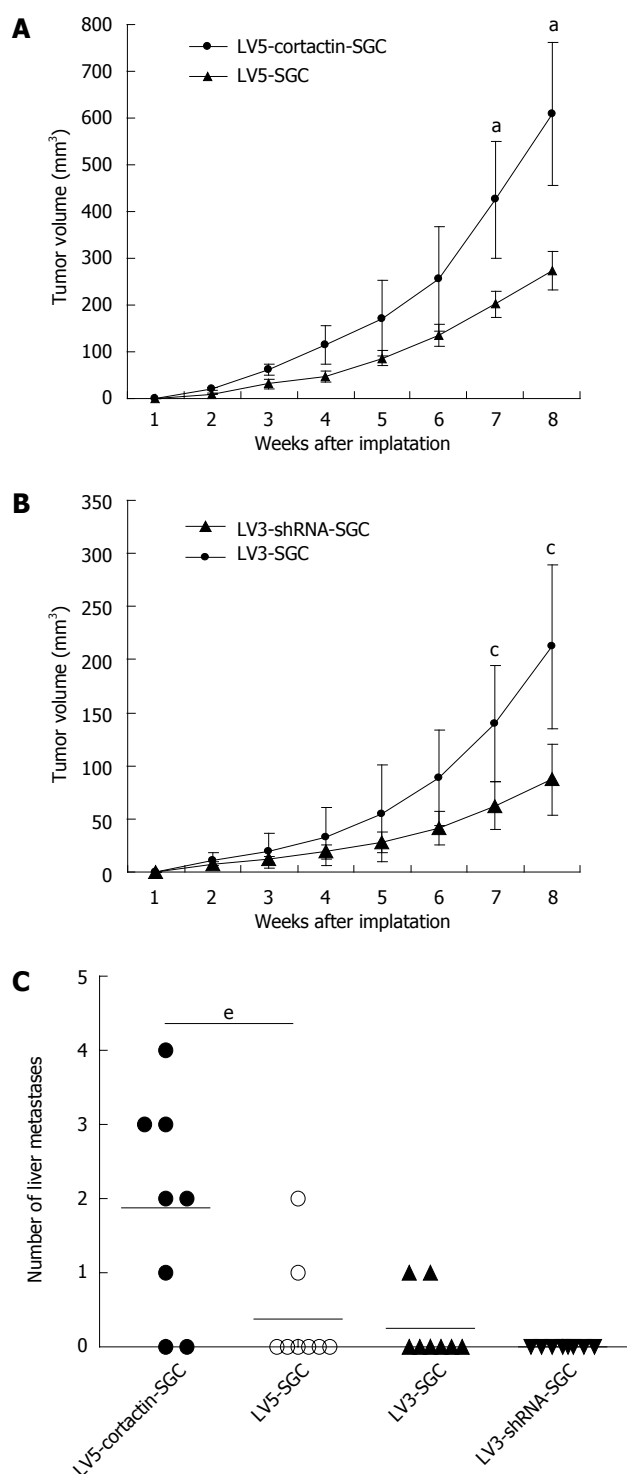


Figure 7 Cortactin expression promotes SGC-7901 cell growth and metastasis *in vivo*. A: Growth curves of the primary tumor volumes (mm³) of the LV5-cortactin-SGC and LV5-SGC cells ($P < 0.05$ vs LV5-SGC cells, Student's *t*-test); B: Growth curves of primary tumor volumes (mm³) of the LV3-shRNA-SGC and LV3-SGC cells ($P < 0.05$ vs LV5-SGC cells, Student's *t*-test); C: Number of liver metastases in the four cell lines ($P < 0.05$ vs LV5-SGC cells, Mann Whitney *U* test).

mo post inoculation. The tumors of the mice inoculated with the LV5-cortactin-SGC cells were obviously larger than the tumors of the mice inoculated with the LV5-SGC cells ($608.2 \pm 153.69 \text{ mm}^3$ vs $274.5 \pm 41.6 \text{ mm}^3$, P

< 0.05 ; Figure 7A). The tumors of the mice inoculated with the LV3-shRNA-SGC cells were smaller than that the tumors of the nude mice inoculated with the LV3-SGC cells (212.5 ± 77.2 vs 87.76 ± 33.6 , $P < 0.05$; Figure 7B). The results suggest that cortactin increases tumor growth *in vivo*. For the spontaneous metastasis assay, the cells were injected into the inferior portion of the gastric serosa of the nude mice. The mice were sacrificed three months after inoculation, and the livers were dissected and analyzed. Six out of 8 mice injected with the LV5-cortactin-SGC cells developed liver metastasis compared with 2 out of 8 mice in the LV5-SGC cell group. In the LV5-cortactin-SGC group, the six mouse livers with metastases harbored 4, 3, 3, 2, 2 and 1 lesions. With regard to the mice injected with the LV5-SGC cells, the two livers of the mice with metastases harbored 1 and 2 lesions ($P < 0.05$, Mann Whitney *U* test; Figure 7C). Two mice developed metastases with 1 secondary tumor in the LV3-SGC group, and no metastasis was noted in the LV3-shRNA-SGC group ($P > 0.05$, Mann Whitney *U* test; Figure 7C). These results indicate that cortactin overexpression induces metastasis *in vivo*. Moreover, cortactin knockdown reduces the metastatic potential of the SGC-7901 cells; however, the results were not statistically significant. The primary and secondary tumors were also confirmed by microscopy (data not shown).

Cortactin promotes the EGFR expression and activates the EGFR signaling pathway in SGC-7901 cells

To study the mechanism by which cortactin enhances the growth of SGC-7901 cells *in vitro* and *in vivo*, the EGFR, STAT3, phosphorylated-STAT3, AKT, and phosphorylated-AKT protein levels were detected by Western blotting. As shown in Figure 8, the levels of EGFR, phosphorylated-STAT3 and phosphorylated-AKT were increased in the LV5-cortactin-SGC cells compared with the LV5-SGC cells, and no difference in the total STAT3 and total AKT protein levels were observed between the two cell groups. The levels of EGFR, phosphorylated-STAT3 and phosphorylated-AKT were decreased in the LV3-shRNA-SGC cells compared with the LV3-SGC cells. Similarly, the expression of total STAT3 and total AKT was not different between the two cell groups. These results indicate that cortactin overexpression enhances the EGFR expression in the SGC-7901 cells and in turn constitutively activates the EGFR signaling pathway.

DISCUSSION

Metastasis remains to be the major cause of treatment failure and poor prognosis in patients with malignant tumors, and it is a multistage process that involves the motility and migration of cells and proliferation in a new site^[22]. Cortactin is a filamentous actin cross-linking protein and a substrate of Src protein tyrosine kinase. The overexpression of wild-type cortactin results in a significant increase in endothelial cell migration^[23]. Cell

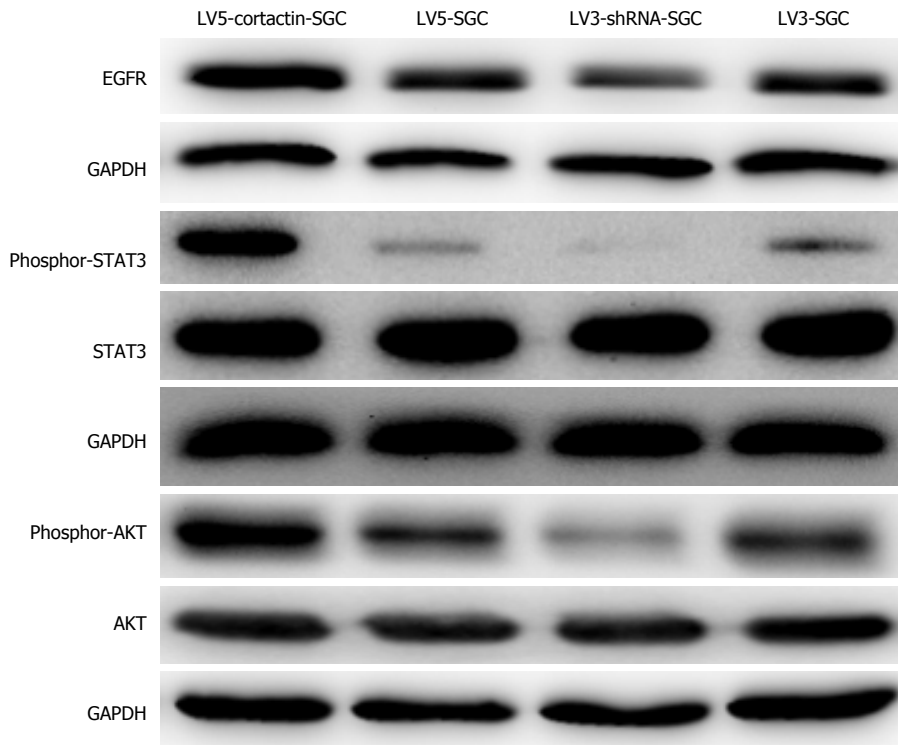


Figure 8 Cortactin expression promotes epidermal growth factor receptor expression and activates the downstream molecules AKT and signal transducer and activator of transcription 3. Western blotting results show that epidermal growth factor receptor (EGFR), phosphor-signal transducer and activator of transcription 3 (STAT3) and phosphor-AKT were increased in LV5-cortactin-SGC cells and decreased in LV3-shRNA-SGC cells compared with their control cells. No alteration in the expression of total STAT3 and total AKT was observed in the four cell lines.

migration is a coordinated process that involves dynamic changes in the actin cytoskeleton and its interplay with focal adhesions^[24]. The cortactin N-terminus associates with F-actin, whereas the C-terminus interacts with focal adhesions^[24]. The phosphorylation of cortactin tyrosine residues by the FAK-Src complex modulates cortactin's interaction with FAK and increases its turnover at focal adhesions to promote cell motility^[24]. Yamada *et al.*^[25] showed that cortactin knockdown results in the down-regulation of adhesion molecules, such as E-cadherin, β -catenin, and epithelial cell adhesion molecule, and leads to the decreased invasion of oral squamous cell carcinoma cells. Nakane *et al.*^[26] showed that cortactin knockdown significantly diminished DU145 cell migration and invasion ability and no apparent morphological changes in the cells were observed by microscopy. Patel *et al.*^[27] showed that NIH3T3 fibroblasts overexpressing cortactin displayed increased motility and invasion in modified Boyden chamber assays without any striking morphological changes. These results are consistent with our observations. As shown in Figures 3 and 4, LV5-cortactin-SGC cell migration and invasion was significantly enhanced compared with the migration and invasion of the LV5-SGC cells, whereas LV3-shRNA-SGC cell migration and invasion was greatly reduced compared with the migration and invasion of the LV3-SGC cells. In addition, no morphological changes were observed in the four cell types (Figure 1). The mechanism that regulates cortactin-mediated effects on the migration

and invasion of cancerous or non-cancerous cells was intensely investigated. One study indicates that cortactin is implicated in the cell-cell adhesion and cell spreading^[28]. Cortactin overexpression results in enhanced cell migration and invasion *via* reduced cell spreading and intercellular adhesive strength^[28]. Hill *et al.*^[29] reported that the cortactin gene was a transcriptional target of the hyaluronan/CD44 standard form signaling, and the mechanistic underpinning of CD44-mediated *EMS1*/cortactin transcription is dependent on a nuclear factor kappa-light-chain-enhancer of activated B cells in breast cancer cells. Cortactin is functionally important in CD44-mediated cell motility and adhesion in endothelial cells^[29]. Clark *et al.*^[30] demonstrated that cortactin expression levels in HNSCC cells correlate with the formation of invadopodia and associated matrix degradation. Moreover, the secretion of the matrix metalloproteinases (MMP) MMP-2 and MMP-9 as well as the surface expression of MT1-MMP is dependent on the level of cortactin expression^[30]. In the cortactin knockdown cells, the number of invadopodia was reduced and these cells were not able to degrade the extracellular matrix (ECM). In the reverse experiment, cortactin overexpression enhanced ECM degradation and invasiveness^[30]. Bryce *et al.*^[31] found that cortactin promotes persistent of lamellipodial protrusion and enhances the rate of new adhesion formation in the lamellipodia through simultaneous interaction with the Arp2/3 complex and actin filaments. These functions are important in cell motility. Rothschild *et al.*^[8] showed

that cortactin tyrosine phosphorylation is an essential requirement for effective HNSCC motility. Another study indicated that tyrosine phosphorylation of cortactin might act as a unique and naive switch in the regulation of cell motility^[32]. The effects of cortactin on cell motility and invasion are becoming increasingly clear and more complex. The present study shows that cortactin phosphorylation was increased in the LV5-cortactin-SGC cells and decreased in the LV3-shRNA-SGC cells compared with the control cells (Figure 2C, D). These results may partly explain the alteration in migration and invasion in the four cell lines, but the specific mechanism requires further elucidation. Clinical studies gave demonstrations that cortactin overexpression is often associated with clinicopathological parameters and poor prognosis in a variety of cancers, such as HNSCC, breast cancer, pancreatic and ampulla of Vater adenocarcinomas, colorectal carcinoma, gastric cancer, non-small cell lung cancer and hepatocellular carcinoma^[11-13,17,33-37]. This association may underscore the important role of cortactin in cell migration and invasion.

To evaluate the effect of cortactin expression on cell proliferation, the MTT assay, colony formation assays and flow cytometry were performed. The MTT assay (Figure 5A) and colony formation assay (Figure 5C, D and G) results showed that cortactin overexpression increased the proliferation of the LV5-cortactin-SGC cells compared with the LV5-SGC cells. LV3-shRNA-SGC cell proliferation significantly reduced compared with that of the LV3-SGC cells as assessed by the MTT assay (Figure 5B) and the colony formation assay (Figure 5E, F and H). Flow cytometry analysis demonstrated that cortactin overexpression increased the proliferation index of the LV5-cortactin-SGC cells compared with that of the LV5-SGC cells (Figure 6A, B and E). Cortactin downregulation impaired the proliferation index of LV3-shRNA-SGC cells compared with that of LV3-SGC cells (Figure 6C, D and F). The Western blotting results showed that cortactin overexpression promoted EGFR expression, and cortactin downregulation impaired EGFR expression. These results are consistent with a previous study showing that cortactin overexpression is associated with attenuated ligand-induced EGFR down-regulation in HeLa cells^[18]. Moreover, RNAi-mediated reduction of cortactin expression accelerates EGFR degradation in HNSCC cell lines^[18]. The molecules downstream of EGFR were also detected by Western blotting, and the levels of phosphorylated-STAT3 and phosphorylated-AKT were increased in the LV5-cortactin-SGC cells and decreased in the LV3-shRNA-SGC cells compared with their control cells. The levels of total STAT3 and total AKT were similar among the four cell types. These results directly support the notion that cortactin overexpression is implicated in EGFR upregulation and constitutive downstream signaling, thereby conferring a proliferation advantage to SGC-7901 cells. EGFR is frequently implicated in cancer cell proliferation, the inhibition of apoptosis and tumor-induced neovascularization *via* the activation of downstream signaling pathways, in-

cluding the PI3K/Akt, the MAPK and the Jak2/STAT3 pathways. EGFR is often overexpressed in various types of tumors, such as salivary gland carcinomas, colorectal cancer (CRC), non-small-cell lung cancer (NSCLC), biliary tract cancer, and gastric cancer^[38-43]. Galizia *et al.*^[43] showed that EGFR overexpression is correlated to poor prognosis in gastric cancer. However, Kim *et al.*^[44] showed opposite effects. Though the role of EGFR expression in gastric cancer prognosis is not clear, the present study demonstrated that EGFR is involved in the progression of gastric cancer. In addition to the ability to migration to secondary sites, the potential to grow from micrometastasis to a macrometastasis is an important factor in the metastatic process. Our mouse tumor growth and metastasis assay also confirmed that cortactin overexpression promotes the growth and metastasis of SGC-7901 cells *in vivo*. These more malignant phenotypes may be attributed to the overexpressions of cortactin and EGFR, which confer migration, invasion and proliferation advantages to SGC-7901 cells both *in vivo* and *in vitro*.

EGFR targeted therapy has been applied in various cancers, including NSCLC, HNSCC and CRC^[41,45,46]. This treatment strategy is currently being explored in clinical trials for gastric cancer patients^[47]. Though current EGFR targeting agents may produce dramatic responses, these drugs are effective in only a fraction of patients. Moreover, resistance to these agents frequently develops^[40]. Therefore, effective treatments may involve a combination of different targeted agents or chemotherapeutic compounds. Based on the present study, cortactin may be a promising coupled target for gastric cancer in the future.

In conclusion, cortactin expression confers a more malignant phenotype to SGC-7901 cells both *in vitro* and *in vivo*. Cortactin expression upregulates EGFR expression, and cortactin and EGFR synergistically contribute to the progression of gastric cancer. Cortactin may serve as a novel therapeutic target for gastric cancer. The role of cortactin in the progression of gastric cancer warrants further studies.

COMMENTS

Background

Gastric carcinoma is one of the most common cancers in the world and the second leading cause of cancer death worldwide. Studies have shown that cortactin overexpression directly correlates with more advanced cancer and lymph node stages, the degree of differentiation and poor prognosis in gastric cancer. However, the mechanism by which cortactin affects gastric cancer progression remains largely unknown.

Research frontiers

Cortactin was first identified in chicken cells transformed by the src oncogene. Cortactin is involved in the progression of a variety of cancers, such as head and neck squamous cell carcinoma, breast cancer, pancreatic and ampulla of Vater adenocarcinomas, colorectal carcinoma, hepatocellular carcinoma, non-small cell lung cancer and gastric cancer. This research aims to clarify the effects of cortactin carcinogenesis.

Innovations and breakthroughs

Previous studies indicated that cortactin is associated with clinicopathological parameters and poor survival in gastric cancer. In this study, we investigated the role of cortactin in the development of the disease. The authors found

that cortactin expression promotes the migration, invasion and proliferation of SGC-7901 cells both *in vivo* and *in vitro*. Additionally, cortactin upregulates epidermal growth factor receptor (EGFR) expression in gastric cancer cells. Cortactin and EGFR contribute synergistically to gastric cancer progression.

Applications

The study indicates that cortactin may serve as a novel therapeutic target for gastric cancer in the future.

Terminology

Cortactin protein was first identified in chicken cells transformed by the src oncogene. Cortactin is an actin-related protein 2/3 complex-activating and filamentous (F)-actin-binding protein that is implicated in tumor cell motility and metastasis. In carcinoma cells that constitutively overexpress cortactin, this protein accumulates in the cytoplasm as well as protruding leading lamellae or podosome-like structures, thereby contributing to the invasive potential of these tumor cells.

Peer review

The manuscript is conducted according to the highest standards of biomedical research. Furthermore, it addresses the topic of molecular biology of gastric cancer and clearly demonstrates the effects of cortactin on its progression, invasion and metastasis that matter on national and international scale. Authors represent a comprehensive study on the effects of cortactin on tumor biology of SGC-7901 cells and identify the mechanism involved in the process. He has no question with this paper and he highly recommends publication.

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Regulatory effect and mechanisms of carbon monoxide-releasing molecule II on hepatic energy metabolism in septic mice

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Abstract

AIM: To investigate the possible mechanisms of exogenous carbon monoxide-releasing molecule II (CORM-2) intervention on hepatic energy metabolism in experimental sepsis.

METHODS: Forty-eight C57BL/6 mice were randomly divided into four groups ($n = 12$): sham group; cecal ligation and puncture (CLP) group; CLP + CORM-2 group and CLP + iCORM-2 (inactive CORM-2) group. Survival rates were determined after 72 h. Twenty-four similarly treated mice ($n = 6$ in each group) were assayed for post-operative continuous blood glucose in the first 36 h. Thirty-six similarly treated mice ($n = 9$ in each group) underwent micro-positron emission tomography (PET) scanning after tail vein injection of ^{18}F -fluorodeoxyglucose (FDG) 24 h after operation. Plasma and liver specimens were collected for assay of liver

pathology, alanine transaminase (ALT) and aspartate transaminase (AST) activities. Hepatic glucokinase activity, lactic acid levels and mitochondrial swelling were also determined.

RESULTS: Improved survival was observed in CORM-2 treated mice. Both the CLP and CLP + CORM-2 groups had sustained low blood glucose levels within the first post-operative 36 h. ^{18}F -FDG micro-PET images showed abnormally high levels of hepatic glucose metabolism (standardized uptake value) in the CLP group (2.76 ± 0.39 vs 0.84 ± 0.14 , $P < 0.01$), which declined to normal levels after CORM-2 intervention (1.29 ± 0.32 vs 2.76 ± 0.39 , $P < 0.05$). glucokinase activity was markedly increased in the CLP group (6.38 ± 0.56 U/g vs 4.60 ± 0.21 U/g, $P < 0.01$), but was normal after CORM-2 intervention (4.74 ± 0.14 U/g vs 6.38 ± 0.56 U/g, $P < 0.05$). CORM-2 suppressed plasma lactic acid levels (4.02 ± 0.02 mmol/L vs 7.72 ± 2.37 mmol/L, $P < 0.05$) and protected hepatic mitochondria in CLP mice. CORM-2 intervention also reduced elevated plasma AST (199.67 ± 11.08 U/L vs 379.67 ± 16.34 U/L, $P < 0.05$) and ALT (63.67 ± 12.23 U/L vs 112.67 ± 9.74 U/L, $P < 0.05$) activities in CLP mice.

CONCLUSION: The release of CO molecules by CORM-2 protects mitochondria and maintains a stable level of hepatic glucose metabolism. Thus, CORM-2 improves liver function and survival in septic mice.

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Key words: Sepsis; Carbon monoxide; Liver; Energy metabolism; Regulation

Core tip: This study used an exogenous carbon monoxide (CO) intervention for the first time and ^{18}F -fluorodeoxyglucose/micro-positron emission tomography to detect hepatic glucose metabolism *in vivo* in septic

mice. The protective effect of CO on hepatic mitochondria in septic mice was examined, and its regulatory effect on abnormal glucose metabolism was explored. The results will provide new evidence for potentially improving outcomes as a consequence of exogenous CO on the survival rate in septic patients.

Liang F, Cao J, Qin WT, Wang X, Qiu XF, Sun BW. Regulatory effect and mechanisms of carbon monoxide-releasing molecule II on hepatic energy metabolism in septic mice. *World J Gastroenterol* 2014; 20(12): 3301-3311 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i12/3301.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i12.3301>

INTRODUCTION

Sepsis is a systemic inflammatory response caused by infections, trauma, and various diseases. It has become the most common cause of death in modern clinics^[1]. Severe pathological changes associated with sepsis are present throughout the development process from the systemic inflammatory response syndrome to the multi-organ dysfunction syndrome^[2]. Despite extensive studies of the inflammatory changes on the occurrence and development of sepsis, less is known regarding the changes in energy metabolism in vital organs associated with sepsis.

Sepsis is generally considered to involve physiological changes in the body towards high metabolism. Characteristic changes include increased energy consumption, excessive protein and fat metabolism, negative nitrogen balance, high blood glucose (hyperglycemia) levels, and excessive production of glycogen^[3,4]. To date, there is no consensus on the cause of high metabolism. This complex response is jointly influenced and determined by a series of factors, including endotoxin, interleukins (IL-1 and IL-6), platelet-activating factor, tumor necrosis factor, arachidonic acid (metabolite of the cyclooxygenase and lipoxygenase pathways) metabolism, reactive oxygen species, neutrophil adhesive material, nitric oxide, complement, and the coagulation cascade^[5]. Overall, high blood glucose is an important causative factor of enhanced metabolism in sepsis. Early onset and sustained development of hyperglycemia are possibly important factors responsible for the high mortality seen in sepsis^[6].

Studies have shown that oxidative decomposition of glycogen is restricted in patients with sepsis, and the resulting hyperglycemia is closely related to the manifestations of septic patients regarding disease progression, immune dysfunction, poor wound healing, and enhanced muscle protein breakdown^[6-11]. In addition, the survival rate of patients with severe acute sepsis can be improved significantly by strengthening the application of insulin to control blood glucose to near normal levels^[12]. However, a recent large randomized study showed that the control of blood glucose levels by intensive insulin therapy cannot effectively reduce the mortality rate of patients in intensive care units (ICU), and instead increases the risk

of low blood glucose (hypoglycemia) and hypoglycemia-induced complications in critically ill patients^[13-15]. Therefore, it is crucial to find a new and effective mode of treatment as a secondary or even alternative option to insulin therapy in maintaining normal blood glucose levels in septic patients.

Carbon monoxide (CO) is one of the metabolic products of heme oxygenase (HO), and regulates inflammation. Our previous studies have demonstrated that endogenous CO has a protective effect on vital organs (including liver, lung, and intestine) by suppressing multiple organ inflammatory responses and reducing oxidative stress^[16-20]. No other study has thus far assessed the regulatory effect of CO on glucose metabolism in sepsis.

In the present study, an exogenous CO intervention was used for the first time and ¹⁸F-fluorodeoxyglucose (FDG)/micro-positron emission tomography (PET) was employed to detect hepatic glucose metabolism *in vivo* in septic mice. The protective effect of CO on hepatic mitochondria in septic mice was examined, and its regulatory effect on abnormal glucose metabolism was explored. The results will provide new evidence for potentially improving outcomes as a consequence of exogenous CO on the survival rate in septic patients.

MATERIALS AND METHODS

Materials

Tricarbonyldichlororuthenium (II) dimer (CORM-2) was obtained from Sigma Aldrich (St Louis, MO, United States) and solubilized in dimethyl sulfoxide (DMSO) to obtain a 40 mmol/L stock. The inactive form of CORM-2 (negative controls) was used in the experiments and was prepared as follows: CORM-2 was "inactivated" (iCORM-2) by leaving the CORM-2 stock at 37 °C in a 5% CO₂ humidified atmosphere for 24 h to liberate CO. The iCORM-2 solution was also bubbled with nitrogen to remove the residual CO present in the solution. An adenosine triphosphate assay kit and a bicinchoninic acid protein assay kit were obtained from Beyotime (Jiangsu, China). A lactate assay kit was obtained from Jiancheng (Jiangsu, China). A blood glucose meter was purchased from ACCU-CHEK Performa (Roche, Germany). ¹⁸F-FDG was obtained from Jiangsu Institute of Nuclear Medicine (Jiangsu, China). A small animal PET device and 3D OSEM MAP imaging system were purchased from Inveon Dedicate (Siemens, Germany). A WIZARD Gamma Counter was obtained (PE/1470 Wizard, United States).

Animal model of cecal ligation and puncture

C57BL/6 male mice (20 ± 2 g) were randomly divided into four groups, including the sham group which received no treatment, the cecal ligation and puncture (CLP) group which underwent CLP surgery, the CLP + CORM-2 and CLP + iCORM-2 group which underwent CLP surgery, followed by tail vein injection of 8 mg/kg CORM-2 and iCORM-2, respectively. The concentration of CORM-2 injected into mice was obtained from

the literature and relevant experience of our research group^[16,17,21]. The Council on Animal Care and Use at Jiangsu University approved the experimental protocol on animal protection and welfare. Anesthesia consisted of the spontaneous inhalation of isoflurane-N₂O in a 60% oxygen/40% nitrogen mixture, which was performed when necessary.

Measurement of mouse survival rate after CLP

A total of 48 C57BL/6 male mice (aged 6–8 wk and weighing 20 ± 2 g) were fed in the laboratory for 1 wk. The mice were divided into four groups ($n = 12$) according to a random number table as described above. Mouse survival was monitored six times daily for up to 72 h.

Blood glucose measurements

A total of 24 C57BL/6 male mice (aged 6–8 wk and weighing 20 ± 2 g) were randomly divided into four groups ($n = 6$) and treated as described above. Blood glucose levels were assayed in all groups of mice preoperatively, and at 1, 2, 4, 6, 8, 10, 12, 16, 20, 24 and 36 h postoperatively. During glucose measurement, the tail tip (2–3 mm) was excised and massaged to harvest a small volume of blood (1.0–10 μ L) which was placed into the hole of a blood glucose test strip. Glucose measurements were obtained using a rapid blood glucose meter. For continuous monitoring, blood flow was obtained by scrubbing the end of the tail with sterile-clean alcohol wipes.

Preparation of liver tissue homogenates

A total of 36 C57BL/6 male mice were randomly divided into four groups ($n = 9$) and treated as described above. All mice were sacrificed 24 h postoperatively. Plasma and complete liver tissue specimens were collected and stored in liquid nitrogen at -70°C before use. To prepare tissue homogenates, 0.1 g specimens were weighed and added to 1 mL of phosphate buffered saline solution and processed by ultrasonic lysis on ice twice (30 s each)^[16,17]. The homogenates were centrifuged at 12000 g and 4°C . The supernatants were collected and stored at -70°C before use.

Histopathological examination

Liver tissue specimens (approximately 0.4 g) were taken from the left lobe of the liver 24 h postoperatively and fixed in 10% formalin, followed by routine paraffin embedding and sectioning. After hematoxylin/eosin (HE) staining, the sections were observed under a light microscope ($\times 400$ magnification) to examine sinusoidal congestion, cellular edema, and inflammatory cell infiltration in liver specimens^[22].

Micro-PET imaging and ^{18}F -FDG biodistribution

During the testing procedures, continuous anesthesia was achieved by mask inhalation of isoflurane with a VMR small animal anesthesia machine. ^{18}F -FDG was administered by tail vein injection at a dose of 20 μ Ci. Micro-

PET scanning was performed after 60 min of continuous anesthesia, during which attention was paid to temperature control and anesthetic dose. Continuous scanning was performed with a small animal PET device for 10 min, and images were reconstructed using a 3D OSEM MAP imaging system. The obtained data were processed by free attenuation correction using the β -smoothing parameter of 0.1. The maximum uptake value of FDG in the liver of each mouse was calculated by measuring the ^{18}F radioactivity in the marked contour area of the liver. The standardized radioactive uptake value (SUV) in each mouse was obtained by standardization of tissue weight and calculation of the injection decay time of the radioisotope^[23]. Mice were euthanized immediately after micro-PET scanning. Complete liver specimens were weighed and the radiation level was then detected with a γ -ray counter. The calculated results were standardized to obtain the final SUV values^[24,25].

Transaminase, glucose-metabolizing enzyme, and lactic acid assays

Blood specimens were collected by right ventricular puncture. Plasma was separated and stored at 4°C in heparin tubes. Plasma alanine transaminase (ALT) and aspartate transaminase (AST) activities were assayed using an automatic blood biochemical analyzer. Hepatic glucokinase (GK) activities and lactic acid levels were assayed by enzyme-linked immunosorbent assay kits following the manufacturer's instructions.

Detection of mitochondrial function

Hepatic mitochondria were isolated by the Aprille method^[26]. Mitochondria were resuspended in the assay buffer which was supplemented with 125 mmol/L sucrose, 50 mmol/L KCl, 2 mmol/L KH₂PO₄, 5 μ mol/L rotenone, 10 mmol/L HEPES, and 5 mmol/L succinate with a protein content of 1.0 mg/mL. The extent of mitochondrial swelling was assayed by measuring the decrease in absorbance (A540) every 30 s for 30 min following the addition of 50 μ mol/L Ca²⁺ at 37°C .

Statistical analysis

Measured data are presented as a mean value \pm SD. Statistical analysis of the experimental data was performed by single-factor one-way analysis of variance using SPSS 17.0 software and independent specimen Student's t test. Survival analysis was performed by log-rank sum test. An alpha value of $P < 0.05$ was considered a statistically significant difference between measures and outcomes.

RESULTS

Survival of septic mice

In this study, the 72 h survival rate of mice in each treatment group was examined postoperatively. The results showed that the survival rate of mice in the CLP group was substantially low, and was only 42% at 24 h and 26% at 48 h postoperatively. In contrast, the survival rate of

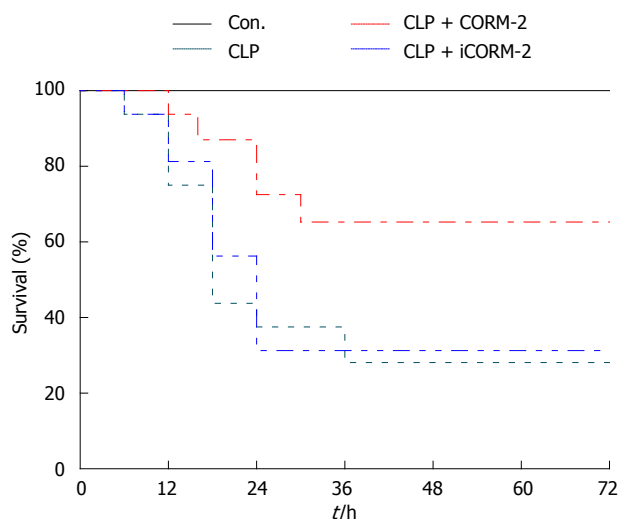


Figure 1 Effect of carbon monoxide-releasing molecule II on survival of septic mice. Mice were challenged with cecal ligation and puncture (CLP) and treated with tricarbonyl-dichlororuthenium (II) dimer (CORM-2) or iCORM-2 as described in the Methods section. All mice had access to water and food. Mouse survival was monitored six times daily for up to 72 h after surgery. Data are shown as the percentage of surviving animals. The survival rate of mice in the CLP group was substantially low, and was only 42% at 24 h and 26% at 48 h postoperatively. In contrast, the survival rate of mice in the CORM-2 group was significantly increased after the CORM-2 intervention, and was 73% at 24 h and 66% at 48 h postoperatively ($P < 0.05$). Moreover, at 72 h postoperatively, 66% of the mice were still alive.

mice in the CORM-2 group was significantly increased after the CORM-2 intervention, and was 73% at 24 h and 66% at 48 h postoperatively ($P < 0.05$). Moreover, at 72 h postoperatively, 66% of mice were still alive (Figure 1).

Effect of CORM-2 on blood glucose in septic mice

In each experimental group, blood glucose levels decreased to 30%-50% of that found in normal controls, and remained at these low levels for more than 36 h postoperatively ($P < 0.05$). In addition, substantial changes in blood glucose levels were not found in the CLP mice after CORM-2 intervention ($P > 0.05$, Figure 2).

Histopathological changes in the liver of septic mice

Under light microscopy, liver specimens from mice in the CLP group showed sinusoidal dilatation and congestion, hepatocellular swelling, vacuolar changes in partial hepatocytes, and neutrophil infiltration. In the CLP + CORM-2 group, sinusoidal congestion and hepatocellular swelling remained in certain areas of the liver specimens, however, hepatocellular damage was less severe and the extent of hepatic neutrophil infiltration was reduced (Figure 3).

Effect of CORM-2 on hepatic glucose metabolism in septic mice

In vivo FDG/micro-PET imaging showed the uptake of the radioactive tracer in the experimental groups 1 h after tail vein injection. The marked area represents the coronal section of a liver from a septic mouse. Moreover, the greater the brightness of this area, the higher the FDG

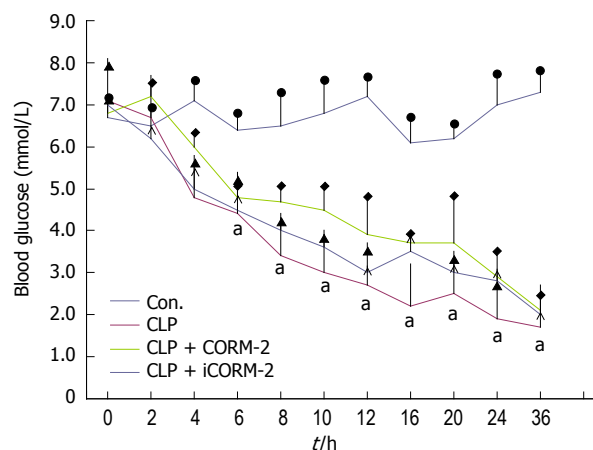


Figure 2 Effect of carbon monoxide-releasing molecule II on blood glucose in septic mice. Mice were challenged with cecal ligation and puncture (CLP) and treated with tricarbonyl-dichlororuthenium (II) dimer (CORM-2) or iCORM-2 as described in the Methods section. Serum glucose concentrations were assessed in all groups of mice preoperatively, and at 1, 2, 4, 6, 8, 10, 12, 16, 20, 24 and 36 h postoperatively. Blood glucose levels in the CLP group decreased to 30%-50% of the normal controls, and remained at these low levels for more than 36 h postoperatively. However, no changes were found in blood glucose levels in the CLP mice after intervention with CORM-2. Results are mean \pm SD, * $P < 0.05$ vs sham mice.

uptake value of the liver, and thus the greater the ability to take up glucose. Compared with the normal control group, the CLP group of mice showed significant increases in the level of hepatic glucose metabolism, and these increases were suppressed by CORM-2 intervention (Figure 4A and B). After micro-PET scanning, complete liver specimens were extracted for evaluation of ^{18}F -FDG biodistribution and the data obtained quantified the experimental results (Figure 4C).

Effect of CORM-2 on plasma ALT and AST activities in septic mice

Plasma ALT and AST activities are important indicators commonly used to determine the extent of hepatocellular damage. The experimental results showed that the CLP group of mice had significantly increased plasma ALT and AST activities 24 h postoperatively as compared with the sham group ($P < 0.05$). However, the CLP + CORM-2 group had minor increases in plasma ALT and AST activities, and the differences were statistically significant compared with the CLP group ($P < 0.05$, Figure 5). Together, these results indicate that CORM-2 reduces plasma ALT and AST activities in septic mice, thereby mitigating hepatic damage.

Effect of CORM-2 on hepatic mitochondrial function in septic mice

The degree of hepatic mitochondrial swelling was examined to determine sepsis-induced changes in hepatic mitochondrial function. The CLP group of mice had significant hepatic mitochondrial swelling with severely damaged mitochondrial function. After CORM-2 intervention, the extent of hepatic mitochondrial swelling was reduced and mitochondrial function was clearly protected

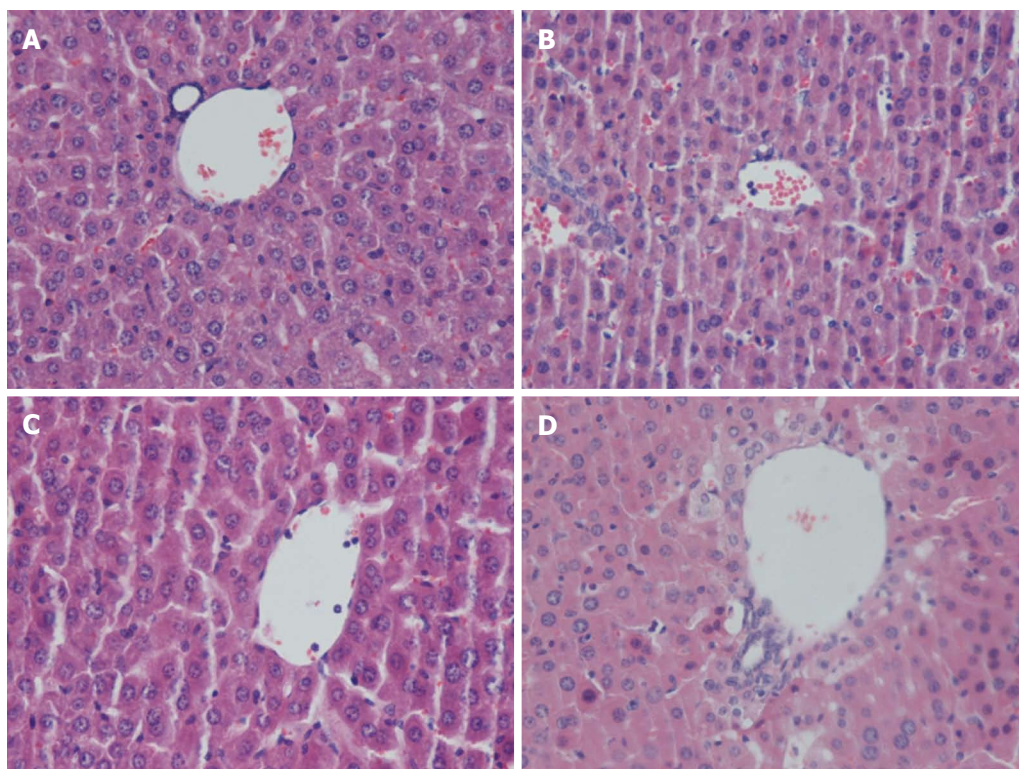


Figure 3 Effect of carbon monoxide-releasing molecule II on liver injury in septic mice. Mice were challenged with cecal ligation and puncture (CLP) and treated with tricarbonyldichlororuthenium (II) dimer (CORM-2) or iCORM-2 as described in the Methods section. Morphologic characteristics at 24 h after CLP were observed under light microscopy with hematoxylin and eosin (HE) staining. Liver specimens from mice in the CLP group showed sinusoidal dilatation and congestion, hepatocellular swelling, vacuolar changes in partial hepatocytes, and neutrophil infiltration. However, in the CLP + CORM-2 group, hepatocellular damage was obviously less severe and the extent of hepatic neutrophil infiltration was reduced. A: Sham; B: CLP; C: CLP + CORM-2; D: CLP + iCORM-2.

($P < 0.05$, Figure 6).

Effect of CORM-2 on hepatic GK activity level in septic mice

GK is a type of hexokinase (HK), and is referred to as HK IV which exists predominantly in the insulin cells of the hepatocyte nucleus. GK is one of the key enzymes involved in hepatic glucose metabolism, and its activity reflects hepatic glucose metabolism. Compared with the normal control group, the CLP group of mice had significantly increased hepatic GK activity. In contrast, the CLP + CORM-2 group had significantly reduced hepatic GK activity (Figure 7).

Effect of CORM-2 on lactic acid levels in septic mice

The levels of lactic acid in plasma and hepatic homogenates of septic mice were determined by enzyme-linked immunosorbent assay. When compared with the sham group, the CLP and CLP + iCORM-2 groups had significantly higher levels of lactic acid ($P < 0.05$). However, after CORM-2 intervention, lactic acid levels in plasma and hepatic homogenates declined significantly (Figure 8).

DISCUSSION

Sepsis is now regarded as one of the leading causes of death in clinics^[27]. According to the hospital statistical

records, millions of septic patients are hospitalized each year in the United States, and there are more than 200000 deaths as a consequence of septic shock^[28]. Sepsis occurs across all age groups in the human population^[29]. It is recognized as the second leading cause of death in ICU patients^[30-32]. The main feature of sepsis is that excessive proinflammatory mediators are released and exceed the ability of the body to physiologically regulate the inflammatory response according to normal homeostatic mechanisms^[33-35]. The reason why a large number of inflammatory factors are produced during sepsis is that multiple vital organs and tissues undergo a variety of pathological changes, including abnormal tissue or cellular metabolism^[36].

The tissue inflammatory response leads to local vasodilation and increases microvascular permeability, with the systemic manifestation in a highly dynamic state. The resulting demand for substrates of energy metabolism (including oxygen, glucose, protein, and fat) is relatively high, which increases energy metabolism and causes a series of clinical manifestations, such as hyperglycemia, lactic acidosis, hyperlipidemia, high protein catabolism, and a negative nitrogen balance. One of the major pathological changes is hyperglycemia. This is caused by an increase in anaerobic glycolysis of muscle tissues and fat metabolism, and a reduction in hepatic glycogen synthesis^[36-38].

In septic patients, hyperglycemia not only means se-

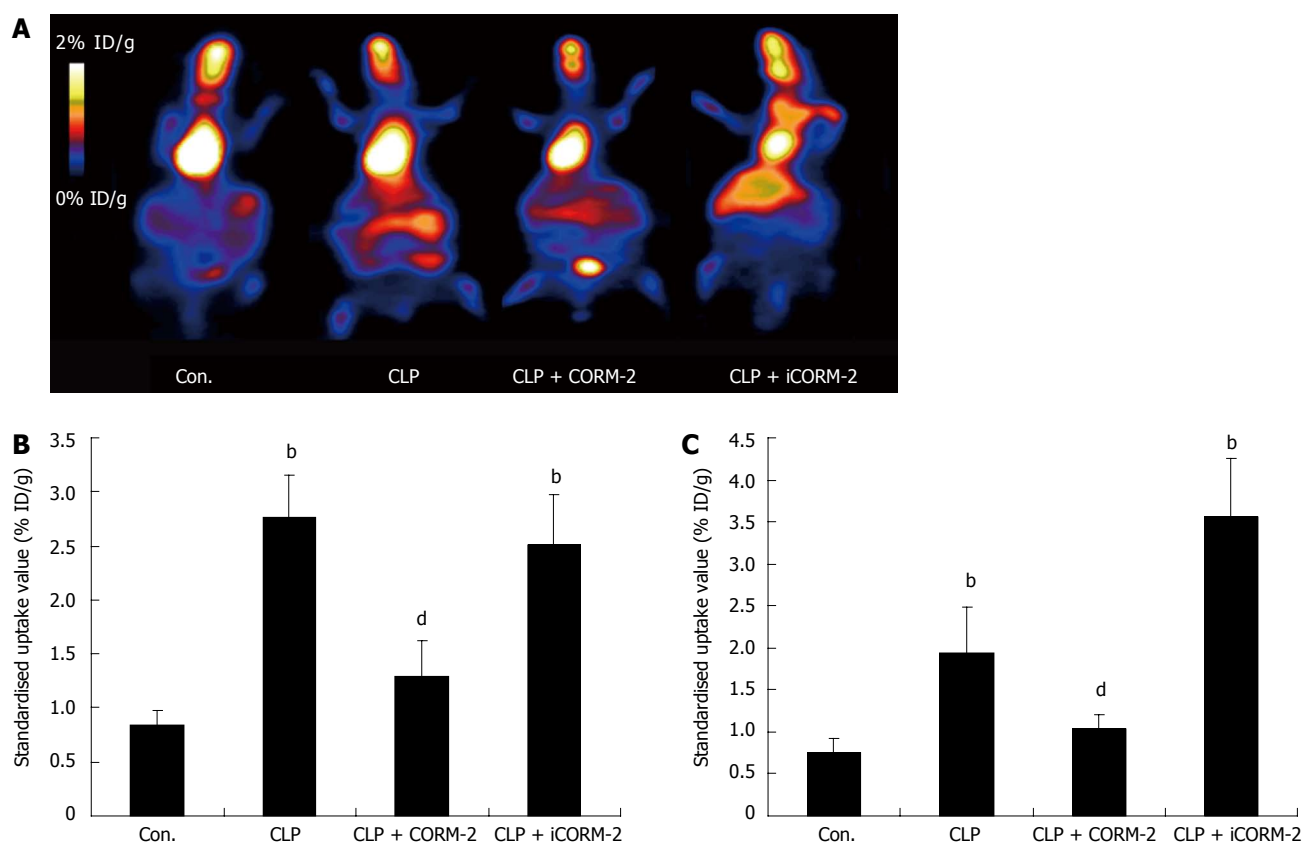


Figure 4 Effect of carbon monoxide-releasing molecule II on fludeoxyglucose uptake in the liver of septic mice. Mice were challenged with cecal ligation and puncture (CLP) and treated with tricarbonyldichlororuthenium (II) dimer (CORM-2) or iCORM-2 as described in the Methods section. ^{18}F -fludeoxyglucose (FDG) was administered by tail vein injection at a dose of 20 μCi followed by micro-positron emission tomography scanning and measurement of ^{18}F -FDG biodistribution. Compared with the normal control group, the CLP group of mice showed significant increases in the level of hepatic glucose metabolism, and these increases were suppressed by CORM-2 intervention. A: Representative images of liver FDG uptake; B: The mean standardized radioactive uptake values in liver tissue; C: Quantification of ^{18}F -FDG biodistribution. ^b $P < 0.01$ vs sham mice; ^d $P < 0.01$ vs CLP mice.

vere illness and a generally poor prognosis^[39,40], but also exerts significant harmful effects on multiple vital organs. Hyperglycemia weakens the immune system, decreases resistance to infection, and reduces neutrophil function (including a reduction in chemotactic ability, the formation of oxygen radicals, and a decreased capacity for phagocytosis of bacteria, despite the actual increase in peripheral vascular leakage). In contrast, hyperglycemia influences the formation of cytokines in tissues, including an increase in proinflammatory cytokine production, such as tumor necrosis factor and IL-6, which are released at an early stage^[41].

Recent studies have shown that blood glucose is not an independent factor which increases the mortality rate in septic patients^[42,43]. However, blood glucose plays a crucial role in severe sepsis and this disease itself has a significant impact on the changes in blood glucose^[44]. Therefore, maintaining stable blood sugar levels is critical in the treatment of sepsis. It is for this reason that a number of studies have attempted to stabilize blood sugar levels in septic patients through intensive insulin therapy at an early stage of treatment, with the aim of improving the survival of patients or reducing their duration of hospitalization^[45]. However, a number of studies have found that such intensive insulin therapy frequently

induces hypoglycemia in septic patients, and serious clinical consequences may arise^[46,47]. In such a dilemma, there is no doubt that a new treatment is needed as a secondary or even alternative approach for the control of blood glucose.

CO is a metabolic product of HO and has a remarkable anti-inflammatory capacity. To date, no study has reported on the effects of CO on the *in vivo* metabolic capacity of glucose or other similar biological agents. Among the metabolic products from the heme portion of HO-1, CO and bilirubin both inhibit apoptosis, necrosis, inflammation, and oxidative stress. Moreover, iron ions enhance the synthesis of anti-oxidants and ferritin. Recent research suggests that the HO system may have a role in insulin sensitivity and cellular metabolism^[48,49].

Recently, transition metal carbonyls have been identified as potential CO-releasing molecules (CORMs) with the potential to facilitate the pharmaceutical use of CO by delivering it to the tissues and organs of interest^[50]. Studies have shown that CORM-2 suppresses LPS-induced inflammatory responses in human umbilical vein endothelial cells (HUVECs), peripheral blood mononuclear cells and macrophages^[51,52]. Similarly, much results have confirmed that CO derived from CORMs protects mice from lethal endotoxemia and sepsis induced by LPS

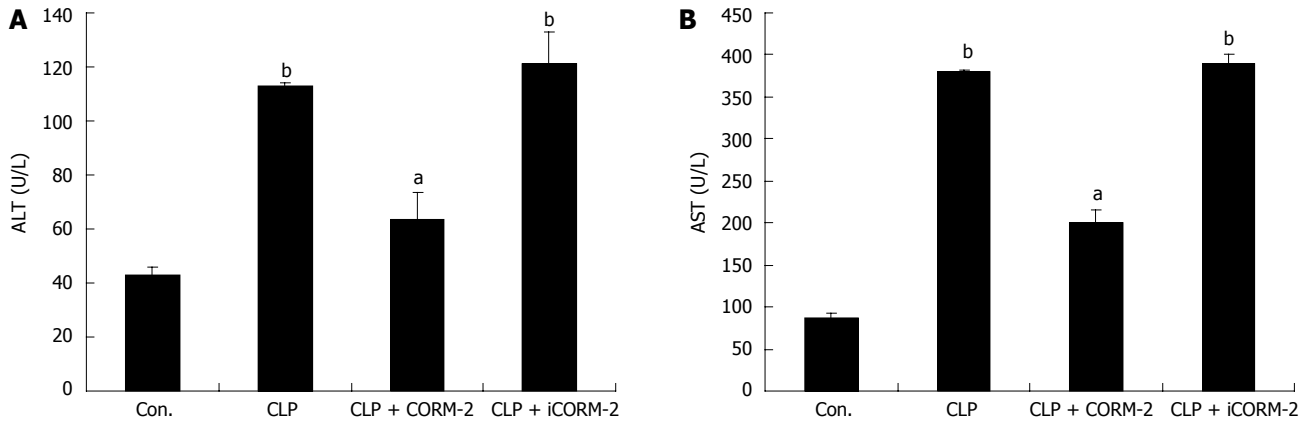


Figure 5 Effect of carbon monoxide-releasing molecule II on plasma aspartate aminotransferase and alanine aminotransferase activities in septic mice. Mice were challenged with cecal ligation and puncture (CLP) and treated with tricarbonyldichlororuthenium (II) dimer (CORM-2) or iCORM-2 as described in the Methods section. Plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities at 24 h after surgery were determined. Plasma activities of ALT (A) and AST (B) were higher in CLP-challenged mice compared with sham mice, whereas ALT and AST activities were markedly decreased after administration of CORM-2. Results are mean \pm SD, ^b $P < 0.01$ vs sham mice; ^a $P < 0.05$ vs CLP mice.

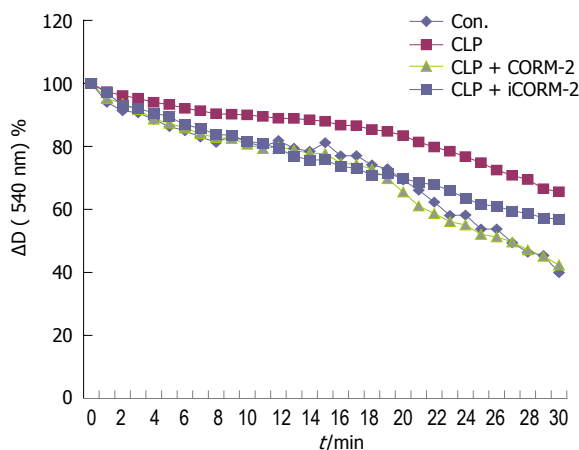


Figure 6 Effect of carbon monoxide-releasing molecule II on hepatic mitochondrial function in septic mice. Mice were challenged with cecal ligation and puncture (CLP) and treated with tricarbonyldichlororuthenium (II) dimer (CORM-2) or iCORM-2 as described in the Methods section. Isolated mitochondria were resuspended in the assay buffer. Ca^{2+} -induced mitochondrial swelling was assayed by the decrease in absorbance at 540 nm. The CLP group of mice had significant hepatic mitochondrial swelling with severely damaged mitochondrial function. Following CORM-2 intervention, the extent of hepatic mitochondrial swelling was reduced and mitochondrial function was clearly protected ($P < 0.05$ vs CLP mice). The curves represent typical recordings from experiments of at least three different mitochondrial preparations.

or cecal ligation and puncture (CLP)^[53-55].

Our previous studies showed that CORM-2 inhibited over-expression of adhesion molecules, attenuated leukocyte sequestration in the organs of CLP or burn-induced septic mice, and decreased intracellular oxidative stress and NO production in LPS-stimulated HUVECs^[16-20]. In the present study, we investigated the changes in hepatic glucose metabolism in septic mice after CLP treatment with or without CORM-2 intervention, and further explored the potential effects of CO on energy metabolism.

The experimental data showed that at 1-2 h after operation, blood glucose levels in the liver of CLP mice significantly decreased to 30%-50% of the normal level,

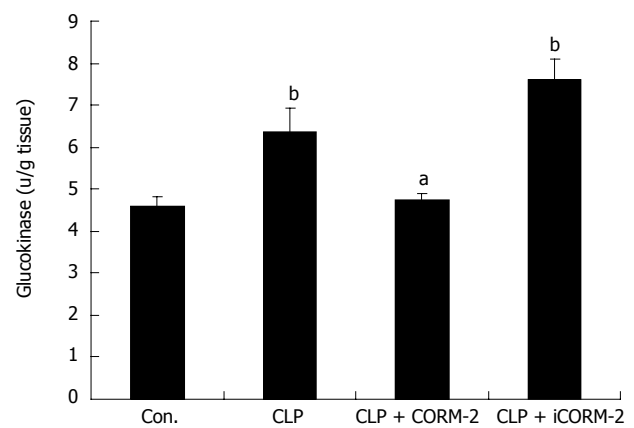


Figure 7 Effect of carbon monoxide-releasing molecule II on hepatic glucokinase activity level in septic mice. Mice were challenged with cecal ligation and puncture (CLP) and treated with tricarbonyldichlororuthenium (II) dimer (CORM-2) or iCORM-2 as described in the Methods section. Hepatic glucokinase activities were assayed by enzyme-linked immunosorbent assay. Compared with the sham group, the CLP group of mice showed a significant increase in hepatic glucokinase (GK) activity. In contrast, the CLP + CORM-2 group showed significantly reduced hepatic GK activity. Results are mean \pm SD, ^b $P < 0.01$ vs sham mice; ^a $P < 0.05$ vs CLP mice.

and stabilized for more than 48 h until death. However, following CORM-2 intervention, blood glucose levels were seen to decrease slightly, but the changes were not statistically significant.

The main reason for this phenomenon may be that the feeding behavior of mice was substantially reduced postoperatively. Since mice are rodents with an inherently active metabolism, the energy required by them after trauma is significantly enhanced. Thus, short-term fasting may cause significant hypoglycemia. Barkhausen *et al.*^[56] reported this phenomenon in 2009. Is this possibly caused by a reduction in metabolic ability after serious infection due to sepsis? If so, then is it contrary to the theory of systemically high metabolism in septic patients studied previously?

Here we investigated *in vivo* hepatic glucose metabo-

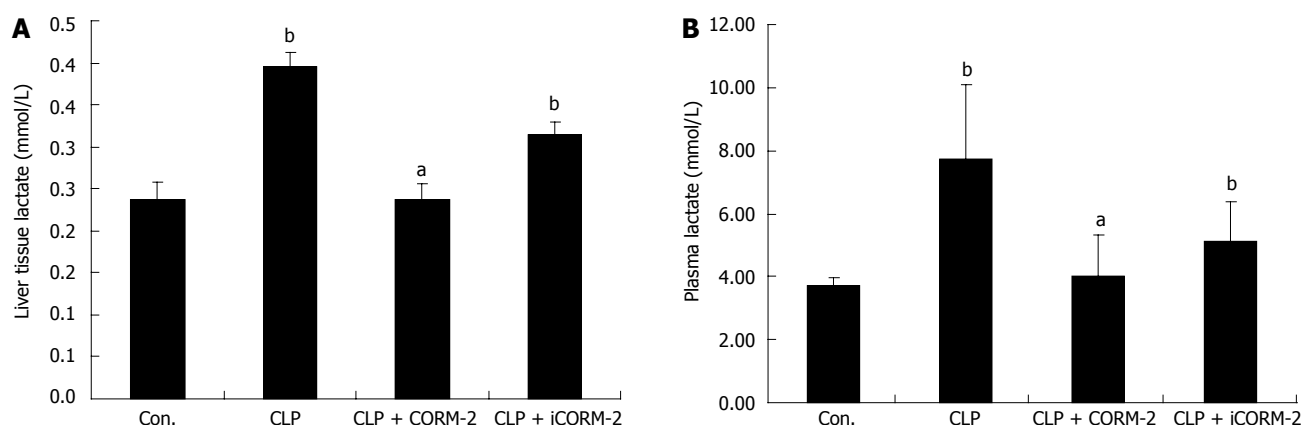


Figure 8 Effect of carbon monoxide-releasing molecule II on hepatic lactate production in septic mice. Mice were challenged with cecal ligation and puncture (CLP) and treated with tricarbonyldichlororuthenium (II) dimer (CORM-2) or iCORM-2 as described in the Methods section. Lactate production in hepatic homogenates (A) and plasma (B) were assessed 24 h after CLP injury. When compared with the sham group, the CLP groups had significantly higher levels of lactic acid. After CORM-2 intervention, lactic acid levels in plasma and hepatic homogenates declined significantly. Results are presented as mean \pm SD. ^b $P < 0.01$ vs sham mice; ^a $P < 0.05$ vs CLP mice.

lism in mice using ^{18}F -FDG/micro-PET. To date, the application of PET technology has enabled remarkable research observations in cancer and neurobiology. In addition, PET technology has been used in gene expression, pharmacological studies, and energy metabolism. However, the application of PET technology in the field of inflammation is restricted, and this is primarily due to the limitations of tracers and resolution. Moreover, research reports to date, have not been available on the application of PET technology in sepsis. This study used micro-PET, which is presently the most advanced molecular imaging technology. To meet the research requirements for model animals of small sizes, PET technology has been improved significantly in terms of spatial resolution and sensitivity.

An increasing number of radiolabels have been used for *in vivo* imaging of small animals. ^{18}F -FDG is a glucose-like radioactive tracer, which has been used for the *in vivo* study of changes in glucose metabolic ability in local tissues and organs in humans and animals, as influenced by various diseases or drugs^[57]. ^{18}F -FDG is transported into cells by the glucose transporter which is located on the cell membrane and then phosphorylated to ^{18}F -2'-FDG-6 by HK. Unlike G-6-P, ^{18}F -2'-FDG-6 does not continue glucose metabolism, but is retained in cells. Therefore, the ^{18}F -FDG levels represent the ability of cells to transport and metabolize glucose. Evidence shows that high FDG uptake not only occurs in malignancies, but is also commonly present in benign inflammatory responses^[58].

In this study, ^{18}F -FDG/micro-PET was used for the first time to investigate changes in hepatic glucose metabolism in septic mice after CORM-2 intervention. The results showed that the level of hepatic glucose metabolism in septic mice significantly increased, and exceeded normal levels. In contrast, CLP mice showed no significant difference when compared with the sham group following CO intervention. In addition, the ^{18}F -FDG biodistribution analysis showed that CO inhibited the abnormal increase in hepatic glucose metabolism in septic mice.

To determine the potential cause of the above phenomena, we determined hepatic GK expression levels in septic mice. GK is primarily found in animal hepatocytes and pancreatic islet B-cells. GK is the key enzyme involved in glucose metabolism and an increase in GK level generally means an elevation in glucose metabolism. In the present study, hepatic GK expression levels in CLP septic mice 24 h after operation, was significantly higher than that seen in the sham group. These changes were obviously reduced after CO intervention. We speculate that CO inhibited the expression of GK, thereby reducing the level of hepatic glucose metabolism in septic mice.

Another important change related to abnormal metabolism in sepsis is lactic acidosis. This process, also known as acid shuttle, results from an increase in anaerobic glycolysis of muscle and other tissues caused by reverse dysregulation of hormones and cytokines. The duration and extent of lactic acidosis directly affects the formation of late onset organ dysfunction, while acidosis caused by lactic acid accumulation is extremely unfavorable in the prognosis of sepsis^[16,36,44].

Interestingly, our experimental data showed that CORM-2 suppressed the abnormal increase in plasma lactic acid levels in septic mice. Lactic acidosis has a significant impact on energy metabolism in the body, and the increase in plasma lactic acid levels is an exact basis for measuring the impairment of mitochondrial function. In this context, we wondered whether CO has a protective effect on hepatocellular mitochondria as it suppressed increases in the levels of plasma lactic acid in septic mice. Hence, we determined the degree of impairment of mitochondrial function by analyzing hepatic mitochondrial swelling in septic mice. The results showed that CO effectively protected hepatic mitochondrial function in septic mice. It is possible that CO regulates blood glucose and plasma lactic acid levels by protecting mitochondrial function.

The protective effect of CO on liver function was

confirmed by observations of hepatic pathological sectioning and serum ALT/AST measurements. CO reduced sinusoidal dilatation and congestion, hepatocellular swelling and deformation, and infiltration of inflammatory cells. In addition, the rise in plasma ALT levels was markedly suppressed after CO intervention.

Liver is the most important organ in the body to regulate blood glucose, and has a substantial ability to regulate glucose metabolism. CO possibly protects liver function in a variety of ways, and thus effectively assists the body to maintain blood glucose metabolism at a normal physiological level. This will significantly favor improvement in the functions of vital organs and tissues, and thus translate to improved prognosis in sepsis. Indeed, this was reflected in our experimental survival analysis data in experimental mice. On the basis of our previous study on the anti-inflammatory effects of CO, the present study opens up a new research direction for exploration of the effects of CO on energy metabolism in the body. This study primarily focused on CO regulation of glucose metabolism and its preliminary mechanism in sepsis. Exogenous CO has significantly physiological functions and could be developed as a potential therapeutic agent. More detailed molecular mechanisms need to be studied further and clarified from a more comprehensive perspective.

COMMENTS

Background

Sepsis is now regarded as one of the leading causes of death in clinics. According to hospital statistical records, millions of septic patients are hospitalized each year in the United States, and there are more than 200000 deaths as a consequence of septic shock. Sepsis occurs across all age groups in the human population. It is recognized as the second leading cause of death in intensive care unit patients. The main feature of sepsis is that excessive proinflammatory mediators are released and exceed the ability of the body to physiologically regulate the inflammatory response according to normal homeostatic mechanisms. The reason that a large number of inflammatory factors are produced in sepsis is that multiple vital organs and tissues undergo a variety of pathological changes, including abnormal tissue or cellular metabolism.

Research frontiers

In septic patients, energy metabolism is increased and causes a series of clinical manifestations, such as hyperglycemia, lactic acidosis, hyperlipidemia, high protein catabolism, and a negative nitrogen balance. One of the major pathological changes is hyperglycemia. This is caused by an increase in anaerobic glycolysis of muscle tissues and fat metabolism, and a reduction in hepatic glycogen synthesis. Hyperglycemia not only means severe illness and a generally poor prognosis, but also has significant harmful effects on multiple vital organs. Hyperglycemia weakens the immune system, decreases resistance to infection, and reduces neutrophil function (including a reduction in chemotactic ability, the formation of oxygen radicals, and a reduced capacity for phagocytosis of bacteria, despite the actual increase in peripheral vascular leakage). In contrast, hyperglycemia influences the formation of cytokines in tissues, including an increase in proinflammatory cytokine production, such as tumor necrosis factor and interleukin-6, which are released at an early stage. Therefore, maintaining stable blood sugar levels is critical in the treatment of sepsis.

Innovations and breakthroughs

In the present study, the authors found that tricarbonyldichlororuthenium (II) dimer (CORM-2), one of the novel CORMs, had a protective role in mitochondria and resulted in a stable level of hepatic glucose metabolism during sepsis. CORM-2 treated mice showed improved survival. Both the cecal ligation and puncture (CLP) and CLP + CORM-2 groups had sustained low blood glucose levels within the first post-operative 36 h. ¹⁸F-fluorodeoxyglucose micro-positron emission tomography images showed abnormally high hepatic glucose me-

tabolism levels in the CLP group, which declined to normal levels after CORM-2 intervention. Glucokinase (GK) expression was markedly increased in the CLP group, but turned normal after CORM-2 intervention. CORM-2 suppressed plasma lactic acid levels and protected hepatic mitochondria in CLP mice. CORM-2 intervention also reduced elevated plasma alanine transaminase and aspartate transaminase levels in CLP mice.

Applications

The protective role of CORM-2 on mitochondria and the level of hepatic glucose metabolism during sepsis, and its therapeutic potential in anti-inflammation will help develop novel strategies against sepsis in the future.

Terminology

Sepsis: it is generally considered to involve physiological changes in the body towards high metabolism. Carbon monoxide (CO): it is one of the metabolic products of heme oxygenase, and regulates inflammation.

Peer review

This is a well designed experimental study of the potential benefit of CO in sepsis. The authors concluded that release of CO molecules by CORM-2 protects mitochondria and maintains the stable level of hepatic glucose metabolism. CORM-2 thus improves liver function and survival in septic mice. Overall, this study is well-done, the paper is clearly written. The reason for the study seems sound. The conclusions seem to be relevant and the literature covers the subject. A few points need to be clarified, but they seem minor.

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Myofibroblastic cell activation and neovascularization predict native liver survival and development of esophageal varices in biliary atresia

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Abstract

AIM: To study the relation between collagen 1, α -smooth muscle actin (α -SMA) and CD34 expression and the most essential portoenterostomy (PE) outcomes.

METHODS: Liver specimens were obtained at PE from 33 biliary atresia (BA) patients for immunohistochemical analysis of collagen 1, α -SMA and CD34. Liver biopsies from 35 organ donors were used as controls. Expression patterns were related to clinical data including age at PE, serum total and conjugated bilirubin concentration at the time of PE and during follow-up, incidence of esophageal varices in follow-up upper gastrointestinal endoscopies, and native liver survival as well as to detailed histopathological findings.

RESULTS: Collagen 1 (16.4% vs 4.5%, $P < 0.0001$), α -SMA (17.9% vs 4.6%, $P < 0.0001$) and CD34 (4.9% vs 3.8%, $P = 0.017$) were markedly overexpressed in BA patients compared with controls. Patients who underwent liver transplantation by age of two years had significantly higher expression of collagen 1 (18.6% vs 13.7%, $P = 0.024$), α -SMA (20.4% vs 15.4%, $P = 0.009$) and CD34 (5.9% vs 4.0%, $P = 0.029$) at PE compared with native liver survivors. CD34-positive microvessels were identified in the centrilobular region close to central vein in every BA patient. In majority of BA cases (56%) neovascularization was frequent as CD34-positive microvessels were observed in over half of the hepatic lobules. In controls, the CD34-positive microvessels were rare as they were completely absent in 40 % and were found in less than 5 % of the hepatic lobules in the rest. The difference between BA patients and controls was significant ($P < 0.0001$). Patients who developed esophageal varices by two years had significantly higher expression of CD34 at PE compared with patients without varices (5.6% vs 4.0%, $P = 0.019$). Expression of α -SMA ($r = 0.758$, $P < 0.0001$) and collagen 1 ($r = 0.474$, $P = 0.016$), and the amount of CD34-positive microvessels ($r = 0.356$, $P = 0.047$) were related to patient age at PE.

CONCLUSION: Hepatic myofibroblastic cell activation, fibrogenesis and neovascularization are enhanced in BA, progress with increasing PE age and relate to native liver survival and development of esophageal varices.

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Key words: Biliary atresia; Liver fibrosis; Neovascularization; Collagen 1; α -smooth muscle actin; CD34

Core tip: The majority of biliary atresia (BA) patients require liver transplantation (LTx) due to progressive hepatic fibrosis and associated portal hypertension.

We aimed to relate expression of collagen 1, α -smooth muscle actin (α -SMA) and CD34 to the most essential portoenterostomy (PE) outcomes. Collagen 1, α -SMA and CD34 were markedly overexpressed in BA patients compared with controls and centrilobular neovascularization was increased in BA. Patients who underwent LTx by age of two years had significantly higher expression of collagen 1, α -SMA and CD34 at PE compared with native liver survivors. Fibrogenesis and neovascularization are enhanced in BA, progress with increasing PE age and relate to native liver survival and development of esophageal varices.

Suominen JS, Lampela H, Heikkilä P, Lohi J, Jalanko H, Pakarinen MP. Myofibroblastic cell activation and neovascularization predict native liver survival and development of esophageal varices in biliary atresia. *World J Gastroenterol* 2014; 20(12): 3312-3319 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i12/3312.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i12.3312>

INTRODUCTION

Biliary atresia (BA) is a rare cholestatic liver disease of infancy leading, if untreated, to cirrhosis, liver failure and death within the first two years of life^[1]. Kasai portoenterostomy (PE) aims to re-establish bile flow and is considered the first line treatment of BA^[2]. However, BA still remains the most common indication for paediatric liver transplantation (LTx) due to progressive liver fibrosis and associated complications such as esophageal varices following PE^[3].

Although the pathogenesis of BA remains unclear, virus-induced fibroinflammatory process affecting the biliary tree is marked^[4,5]. The process of hepatic fibrogenesis and development of cirrhosis is multifactorial and multitude of involving proteins has been identified^[6]. In liver fibrosis extracellular matrix proteins, mainly collagens accumulate excessively. Majority of collagens are deposited by myofibroblasts that differentiate from hepatic stellate cells (HSCs) and portal fibroblasts^[7,8]. In normal liver tissue HSCs are inconspicuous but upon activation these cells transform into α -smooth muscle actin (α -SMA) producing contractile myofibroblastic cells^[9]. In two previous studies with a smaller number of patients, increased α -SMA expression was found to associate with histological fibrosis scores of the liver and PE outcomes in infants with BA^[10,11].

CD34 is a cell surface glycoprotein expressed in vascular endothelium. The relationship between progressive fibrosis and angiogenesis is unclear but neovascularization is found in a variety of liver diseases. This vascular reorganization may represent a response to liver injury including alterations in liver microcirculation associated with portal hypertension and development of esophageal varices^[12,13].

In the present study we explored the hepatic expres-

sion of collagen 1, α -SMA and CD34 at the time of PE in a controlled manner, and related the results to the most essential PE outcomes including clearance of jaundice, native liver survival and development of esophageal varices.

MATERIALS AND METHODS

Patients and study design

This study was performed at the Children's Hospital in Helsinki, Finland. The national paediatric LTx program has been running since 1987 in our hospital and the treatment of BA was nationally centralized to our unit in 2005^[14]. All available perioperative liver biopsies obtained at the time of PE were collected for immunohistological analysis and medical records were reviewed. A total of 33 BA patients born between 1990 and 2012 were enrolled. Collected clinical data included age at PE, serum total and conjugated bilirubin concentration at the time of PE and during follow-up, detection of esophageal varices in follow-up upper gastrointestinal endoscopies, and indications as well as the age at LTx. All patients underwent endoscopic surveillance for esophageal varices as described previously^[15]. A multidisciplinary team (paediatric hepatologists, transplant paediatricians, paediatric gastrointestinal, and transplant surgeons, neurologists) decided on listing for liver transplantation based on the following parameters: (1) original liver disease, quality of life (cholangitis and septic episodes), growth, nutrition, neurology, kidney function, bone health; (2) portal hypertension (portal flow, spleen size, hypersplenism, varices, ascites); (3) cholestasis (bilirubin, bile acid levels, itching); (4) liver biochemistry (ALT, AST, GT, clotting factors, prealbumin, albumin, cholesterol, Galactose half-life); (5) imaging findings (liver and spleen size, parenchymal heterogeneity, biliary lakes, focal lesions); and (6) liver histology (fibrosis, cirrhosis, bile ducts). Clearance of jaundice was defined as a decrease in serum bilirubin concentration below 20 μ mol/L. As controls we examined 35 liver biopsies obtained from deceased donors at organ recovery.

The ethics committee of the hospital district of Helsinki and Uusimaa approved this study a priori and the study conforms to the principles of the 1975 Declaration of Helsinki.

Immunohistochemistry and imaging

Altogether 33 biopsies were taken at the time of Kasai PE, including 6 core needle biopsies and 27 surgical wedge biopsies. The biopsies were fixed in formalin, embedded in paraffin, sliced, and stained with conventional stains. The representativeness of the biopsy material was considered good: > 8 (wedge) or > 10 (needle) portal areas were present in 29 (88%) biopsies. Histological liver fibrosis was assessed by Metavir (0-4) and Ishak (0-6) fibrosis scores by two experienced pediatric liver pathologists, blinded to the clinical patient data, until consensus was reached^[16,17].

Immunostaining for collagen 1 was performed with

Table 1 Prevalence of centrizonal microvessels in biliary atresia patients ($n = 32$) at the time of portoenterostomy and in control ($n = 35$) livers

Number of patients	BA	Controls
Patients		
Grade 1	0	14
Grade 2	5	21
Grade 3	9	0
Grade 4	18	0
	$P < 0.0001$	

Data presented as number of patients. Comparison between groups was performed with Mann-Whitney *U*-test. Microvessel grades: 1: No central zones with microvessels; 2: < 5% of central zones with microvessels; 3: 5%-50% of central zones with microvessels; and 4: > 50% of central zones with microvessels. BA: Biliary atresia.

COL1A2/COL1A1 monoclonal antibody, clone I-8H5 (Abnova Corporation, Taiwan), for α -SMA using Monoclonal Mouse Anti-Human Smooth Muscle Actin, clone 1A4 (Dako, Denmark) and for CD34 using Monoclonal Mouse Anti-Human CD34 Class II, clone QBEnd-10 (Dako, Denmark) and NovoLink Polymer Detection System (Leica Biosystems Newcastle Ltd, Newcastle Upon Tyne, United Kingdom).

A Leica DM RXA microscope was used to obtain images of stained sections. A total of five random portal areas were chosen from each section of wedge biopsies ($\times 100$ magnification) and all core needle biopsies were photographed as a whole. The proportion of the antibody-positive area (area fraction) was measured by using ImageJ Image Analysis Software^[18]. To evaluate neovascularization CD34-positive endothelial cell clusters forming centrizonal microvessels were counted with semi-quantitative scoring system. Each biopsy was reviewed and scored for the presence of microvessels on scale of 1-4 (Table 1). Imaging and all immunohistochemical expression analyses were performed without knowledge of the clinical patient data.

Statistical analysis

The data are reported as means and standard deviation or medians and range. Comparisons between groups were performed with Mann-Whitney *U*-test and multiple comparisons with Kruskal-Wallis test. Correlations were calculated with Spearman's rank correlation. A *P*-value < 0.05 was considered significant. All of the analyses were made with Statview software (Statview 5.0.1; SAS Institute Inc., CA, United States).

RESULTS

Patient characteristics

Table 2 illustrates the patient characteristics at the time of PE. No type I atresias were found and in four patients with type III atresia perioperative cholangiography showed patent passage from gallbladder to bowel (type IIIa). The median (range) age at PE among all BA patients was 64 (7-141) days and median serum bilirubin at

Table 2 Patient characteristics at the time of portoenterostomy

BA type	<i>n</i> (%)	Age at PE (d)	Bilirubin ($\mu\text{mol/L}$)	Conjugated bilirubin
II	3 (9.1)	102 (78-141)	186 (98-247)	162 (86-207)
III	26 (78.8)	64 (7-140)	172 (103-470)	117 (45-224)
IIIa	4 (12.1)	51 (37-81)	171 (103-201)	85 (69-109)
		$P = 0.092$	$P = 0.838$	$P = 0.183$

Data presented as median and range. Comparisons between groups were performed with Kruskal-Wallis test. BA: Biliary atresia; PE: Portoenterostomy.

the time of PE and at three months following PE was 174 (98-470) $\mu\text{mol/L}$ and 21 (2-627) $\mu\text{mol/L}$, respectively. Of the 33 patients, 30 had been followed-up over two years. Overall, 58% (19/33) of the patients cleared their jaundice and 57% (17/30) survived with their native liver beyond two years following PE. By two years after PE, 47% (14/30) of the patients had developed endoscopically verified esophageal varices.

Expression of collagen 1, α -SMA and CD34 in BA vs control livers

As shown in Figure 1, expression of collagen 1, α -SMA and CD34 was markedly increased in BA when compared with controls (16.4% *vs* 4.5%, $P < 0.0001$, 17.9% *vs* 4.6%, $P < 0.0001$, 4.9% *vs* 3.8%, $P = 0.017$, respectively). The increase was most striking for collagen 1 and α -SMA, stained area fractions being about four fold higher than in controls. The portal tracts stained intensively with collagen 1 and marked collagen 1 positive septae protruded towards the central vein. α -SMA staining showed strong immunoreactivity both on portal areas and along perisinusoidal spaces. The expression of collagen 1, α -SMA and CD34 did not differ significantly between different subtypes (II, III, IIIa) of BA ($P = 0.360$, $P = 0.345$ and $P = 0.736$, respectively for collagen 1, α -SMA and CD34).

Expression of collagen 1, α -SMA and CD34 in relation to clearance of jaundice, native liver survival and the age at the time of portoenterostomy

Patients who underwent LTx by age of two years showed significantly higher expression of collagen 1, α -SMA and CD34 in liver biopsies obtained at PE when compared to patients who survived with their native livers beyond two years (Figure 2 and Table 3). Children who cleared their jaundice tended to have lower expression of collagen 1 and α -SMA at PE (Table 3), although this did not reach statistical significance, ($P = 0.079$ and $P = 0.097$, respectively). The degree of α -SMA staining correlated with the level of preoperative conjugated bilirubin ($r = 0.448$, $P = 0.023$), bilirubin level at three months ($r = 0.446$, $P = 0.029$) and relative change of bilirubin level by three months ($r = -0.395$, $P = 0.044$). Patients who underwent LTx by age of two years were older at the time of PE than native liver survivors, but the difference was not quite statistically significant (79.1 d *vs* 65.8 d, $P = 0.054$).

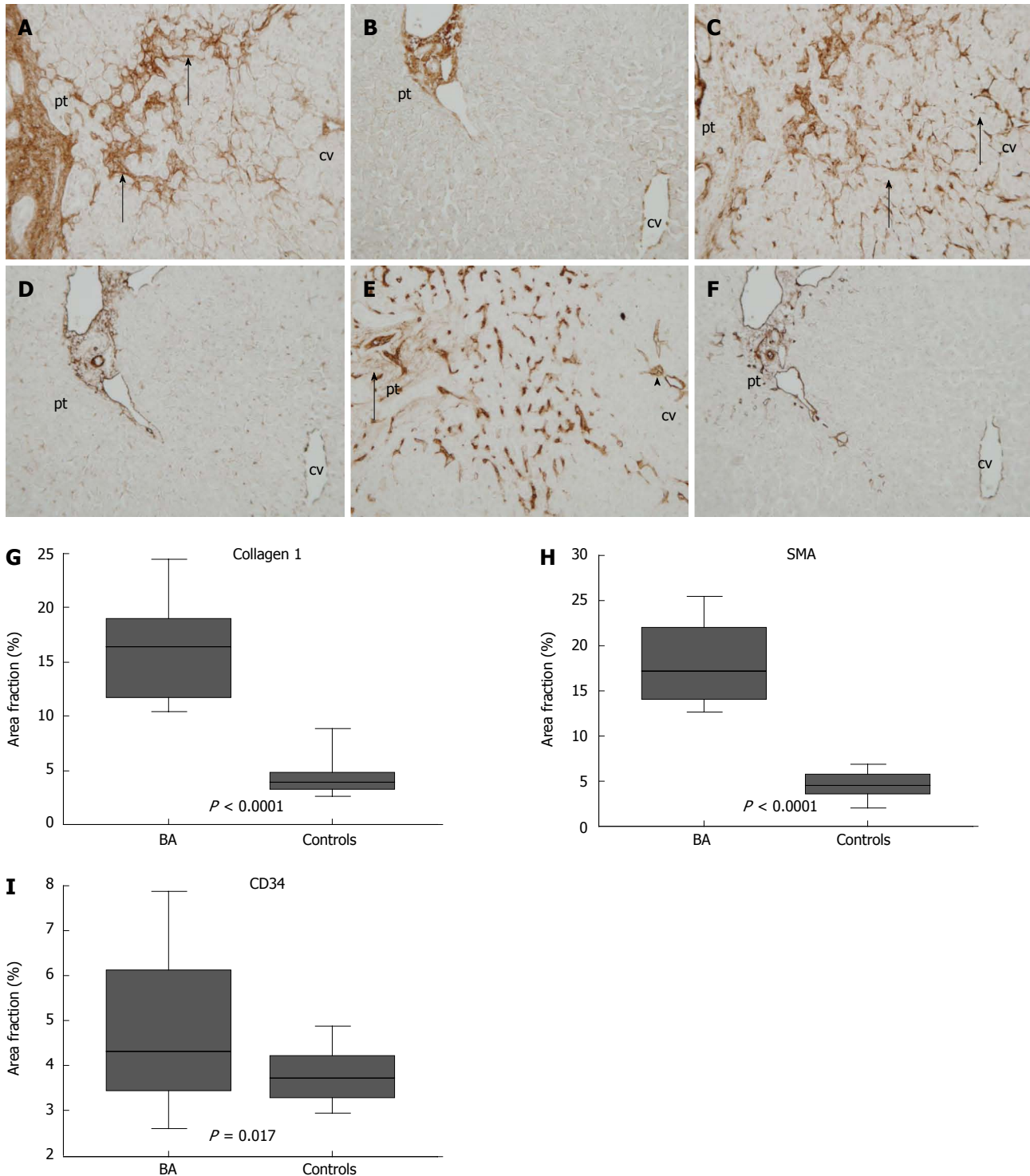


Figure 1 Expression of collagen 1, α -smooth muscle actin and CD34 in biliary atresia vs control livers. Staining of collagen 1 (A, B), α -SMA (C, D) and CD34 (E, F) by immunohistochemistry. Figure A, C and E represent liver biopsies of a BA patient, who underwent Kasai portoenterostomy at the age of 54 d and figure B, D and F are liver biopsies obtained from a deceased donor ($\times 100$ magnification). The same area of the histological tissue section is illustrated after immunohistochemistry for both subjects, respectively. Portal tracts (pt) stain intensively with collagen 1 and marked collagen 1 positive septa (arrows) protrude towards central vein (cv) (A). In control liver collagen 1 staining is limited to portal area and endothelial lining of central vein (B). α -SMA staining shows strong immunoreactivity both in portal areas (pt) and along perisinusoidal spaces (arrows) (C), whereas in control specimen a faint α -SMA staining can be seen in biliary epithelial cells (D). Marked increase in CD34 staining is observed on portal (arrow) and centrilobular (arrowhead) area on liver biopsy of BA patient (E) compared with control (F). Summary of collagen 1 (G), α -SMA (H) and CD34 (I) immunohistochemistry in BA patients and controls. Collagen 1, α -SMA and CD34 were markedly overexpressed in BA at the time of Kasai portoenterostomy compared with controls. The results are expressed as proportion of the antibody-positive area. The box represents interquartile range, line across the box median, and the whiskers 90th percentile range. α -SMA: α -smooth muscle actin; BA: Biliary atresia.

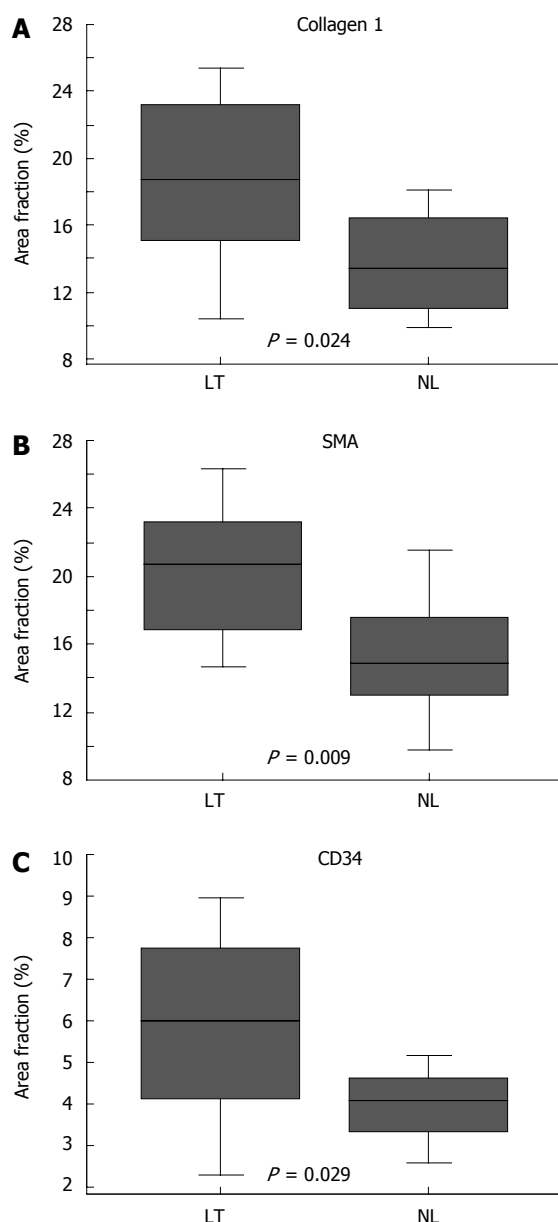


Figure 2 Comparison of collagen 1 (A), α -smooth muscle actin (B) and CD34 (C) staining by immunohistochemistry between patients, who received liver transplant by two years of age and native liver survivors. The results are expressed as proportion of the antibody-positive area. All of the studied proteins were overexpressed at the time of Kasai portoenterostomy in patients, who were transplanted by two years. The box represents interquartile range, line across the box median, and the whiskers 90th percentile range. NL: Native liver; LT: Liver transplant; α -SMA: α -smooth muscle actin.

The age at the time of PE correlated positively with the expression of α -SMA ($r = 0.758$, $P < 0.0001$) (Figure 3), collagen 1 ($r = 0.474$, $P = 0.016$), and the amount of CD34-positive microvessels ($r = 0.356$, $P = 0.047$).

Neovascularization and CD34 immunohistochemistry in relation to esophageal varices

Patients who developed esophageal varices by two years following PE had significantly higher expression of CD34 at the time of PE compared with patients who remained free of varices (Table 3). Of note, expression of collagen 1 or α -SMA were not associated with develop-

ment of esophageal varices.

CD34-positive microvessels were identified in the centrilobular region close to central vein in every BA patient. In 18 BA cases (56%) neovascularization was frequent as CD34-positive microvessels were observed in over half of the hepatic lobules. In controls, the CD34-positive microvessels were rare as they were absent in 40% and were found in less than 5% of the hepatic lobules in the rest (Figure 4 and Table 1). The difference between BA patients and controls was significant ($P < 0.0001$). The amount of CD34-positive microvessels did not differ significantly between different subtypes (II, III, IIIa) of BA ($P = 0.426$).

Interrelations between collagen 1, α -SMA and CD34 and correlations with histological fibrosis scores

Expression of α -SMA was closely related with the expression of collagen 1 ($r = 0.731$, $P = 0.0002$). It also correlated positively with CD34 ($r = 0.502$, $P = 0.011$) expression and histological fibrosis scores (Metavir and Ishak, $r = 0.515$, $P = 0.010$ and $r = 0.511$, $P = 0.011$, respectively). Collagen 1 expression correlated with fibrosis scores (Metavir and Ishak, $r = 0.503$, $P = 0.012$ and $r = 0.513$, $P = 0.010$, respectively) and CD34 expression ($r = 0.625$, $P = 0.0014$).

DISCUSSION

Early diagnosis and successful PE establishing sufficient bile flow are the key events for extended native liver survival in BA^[1]. Even though the majority of BA patients end up with LTx due to progressive hepatic fibrosis and associated portal hypertension it is essential to increase the proportion of BA patients, who survive with their own liver into adolescence or even adulthood. LTx carries well-known risks of allograft ageing and the side effects of long-term immunosuppression^[19]. Several cytokines have been identified to play an important role in the regulation of liver fibrogenesis. Transforming growth factor $\beta 1$ (TGF- $\beta 1$) is one of the most powerful profibrotic cytokines and the family of matrix metalloproteases is recognized as another key mediator in this process^[20,21]. Despite intensive research, understanding of mechanisms that regulate disease progression following PE is scarce. Several candidate genes have been proposed to act as prognostic markers when predicting outcomes^[22,23], but further understanding of pathogenesis of BA is critical concerning development of more effective treatment strategies.

Hepatic stellate cells have a crucial role in the pathogenesis of hepatic fibrosis. After transformation into myofibroblasts in response to a liver injury, stellate cells start to produce increased amounts of collagen and express an intracellular microfilament protein α -SMA, a marker of activated HSC phenotype^[24]. Increased α -SMA expression has been shown to associate with increased liver fibrosis as assessed by histological scoring and PE outcomes in infants with BA^[11,25]. We found a 4-fold increase in α -SMA and collagen 1 expression in BA pa-

Table 3 Hepatic expression of collagen 1, α -smooth muscle actin and CD34 in biliary atresia ($n = 27$) at the time of portoenterostomy and in control ($n = 35$) livers

	Clearance of jaundice		2-yr native liver survival		Esophageal varices		All BA patients	Controls
	Yes	No	NL	LT	Yes	No		
Collagen 1	14.7 (4.9)	18.5 (5.4)	13.7 (3.5)	18.6 (5.4)	17.2 (4.9)	14.6 (5.0)	16.4 (5.4)	4.5 (2.2)
Area, % (SD)	$P = 0.079$		$P = 0.024$		$P = 0.217$		$P < 0.0001$	
α -SMA	16.4 (5.1)	19.8 (4.8)	15.4 (4.9)	20.4 (4.2)	18.2 (4.3)	17.1 (6.1)	17.9 (5.2)	4.6 (1.9)
Area, % (SD)	$P = 0.097$		$P = 0.009$		$P = 0.537$		$P < 0.0001$	
CD34	4.4 (1.4)	5.6 (2.4)	4.0 (0.9)	5.9 (2.4)	5.6 (2.0)	4.0 (1.6)	4.9 (2.0)	3.8 (1.0)
Area, %	$P = 0.213$		$P = 0.029$		$P = 0.019$		$P = 0.017$	

Data presented as mean and SD. Comparisons between groups were performed with Mann-Whitney *U*-test. BA: Biliary atresia; PE: Portoenterostomy; NL: Native liver; LT: Liver transplant; α -SMA: α -smooth muscle actin.

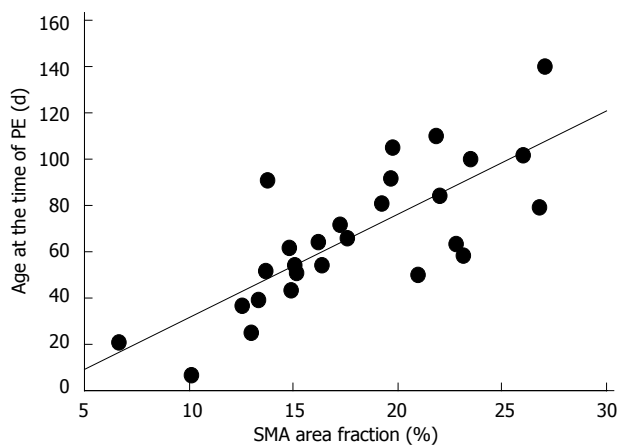


Figure 3 Correlation between the age at the time of portoenterostomy and α -smooth muscle actin expression. A significant correlation was seen using Spearman's rank correlation test ($r = 0.758$, $P < 0.0001$). α -SMA: α -smooth muscle actin; PE: Portoenterostomy.

tients compared with controls. Overexpression of α -SMA staining was related to clearance of jaundice as it correlated with serum bilirubin concentration and relative decrease of bilirubin three months after PE. Patients who survived with their native liver for the first two years had significantly lower expression of α -SMA and collagen 1 at the time of PE than patients who underwent LTx. Those who underwent LTx were older at the time of PE and, although the age difference was not quite statistically significant, it accords well with the improved clinical outcomes of PE performed at early age^[26]. Furthermore, age at PE had a strong positive correlation with the expression of α -SMA and collagen 1. Collectively, these findings suggest that myofibroblastic cell activation associated liver fibrosis progresses with advancing age after birth before PE, being a major predictor of native liver survival.

Besides activated stellate cells, portal myofibroblasts are another cell type expressing α -SMA, and similarly to the present study, increase in α -SMA expression around portal tracts has been previously shown to occur in BA^[27]. Here, an intense increase in α -SMA staining was observed also in perisinusoidal spaces in addition to portal regions. Although definitive source of α -SMA

expressing cells can not be ascertained by immunohistochemistry alone, it seems likely that these cells originated from portal myofibroblasts and activated stellate cells. As expected, expression pattern of accumulating collagen 1 followed that of α -SMA expressing myofibroblastic cells producing extracellular matrix proteins^[7].

CD34 is a glycoprotein expressed on vascular endothelium and CD34 immunostaining can be used to elucidate neovascularization in response to a liver injury^[11,12]. We assessed neoangiogenesis by CD34 staining using both quantitative and qualitative approaches. The degree of CD34 staining was significantly increased in BA patients compared with controls when measured as an immunopositive area on histological liver sections. We then analysed centrilobular areas in hepatic lobules to explore possible neovascularization in the region around the central vein, theoretically most prone to ischemic injury. Only distinct CD34-positive endothelial cell clusters with a vessel lumen were considered as microvessels to avoid misinterpretation of faint diffuse sinusoidal expression of CD34 as revascularization. Microvessels around the central vein were more frequent among BA patients compared with controls, which is a novel finding in BA. Interestingly, patients who developed endoscopically verified esophageal varices during follow-up had significantly higher expression level of CD34 already at the time of PE compared to those who did not. Regarding pathogenesis of esophageal varices, myofibroblastic differentiation of portal fibroblasts and HSC activation lead to increased hepatic vascular resistance by accumulation of extracellular matrix proteins, but also dynamic changes in hepatic vasculature occurring in chronic liver injury are important as evidenced by activation of the renin-angiotensin system^[28]. Experimental studies on portal hypertension and liver cirrhosis further imply that not only altered hemodynamics but also active neovascularization contributes to formation of collateral vessels and that hypoxia and angiogenesis progress along with fibrogenesis in response to chronic liver injury^[29,30]. Hepatopulmonary syndrome (HPS) represents an important cause of pulmonary disease in children with BA. Corresponding animal models suggest involvement of pulmonary neovascularization and activation of angiogenic signalling pathways also in HPS^[31].

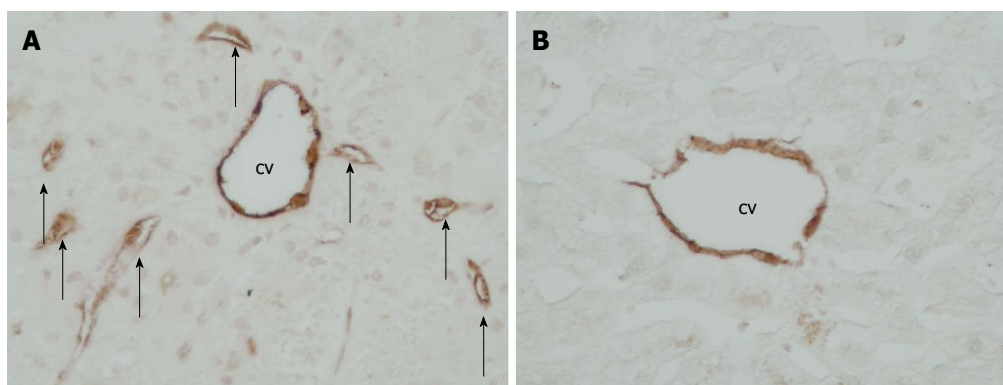


Figure 4 High magnification ($\times 400$) images of CD34 immunohistochemistry on centrilobular region. There are multiple microvessels (arrows) around central vein (cv) in biliary atresia patient who underwent Kasai portoenterostomy at the age of 51 d (A) whereas only endothelium of central vein is stained in control liver (B).

This study has certain limitations such as the relatively small number of patients. It is also impossible to obtain liver biopsies from matched healthy infants. However, considering rarity of BA and current literature our series of 33 BA patients is reasonably good in size.

In conclusion, hepatic expression of α -SMA, collagen 1 and CD34 was increased in BA, suggesting myofibroblastic cell activation associated fibrogenesis and neovascularization. Low expression of α -SMA and collagen 1 at the time of PE was related to improved bile flow and native liver survival. Microvessels were common in the centrilobular region of the hepatic lobules among BA patients already at the time of PE and increased expression of CD34 predicted development of esophageal varices.

COMMENTS

Background

Biliary atresia is a potentially lethal disease affecting extrahepatic biliary tract. It is associated with progressive hepatic fibrosis despite successful primary repair with Kasai portoenterostomy and the pathogenesis of biliary atresia (BA) has remained largely obscure.

Research frontiers

Various biochemical and haematological values at presentation have been shown to relate with the prognosis and the research hotspot has been to understand the factors that predict the outcome after Kasai portoenterostomy.

Innovations and breakthroughs

Low expression of α -SMA and collagen 1 at the time of portoenterostomy (PE) was related to improved bile flow and native liver survival suggesting that myofibroblastic cell activation is an early event in the cascade leading to hepatic fibrosis. Microvessels around the central vein were more frequent among BA patients compared with controls, which is a novel finding in BA.

Applications

The study results suggest that myofibroblastic cell activation associated liver fibrosis progresses with advancing age after birth before PE, being a major predictor of native liver survival.

Terminology

α -SMA is a marker for activated hepatic stellate cells that have a crucial role in the pathogenesis of hepatic fibrosis. CD34 is a glycoprotein expressed on vascular endothelium and CD34 immunostaining can be used to elucidate neovascularization in response to a liver injury.

Peer review

The author examined liver tissue of BA infants at the time of Kasai PE. They found that degree of myofibroblastic cell activation could be a predictive factor for transplant-free survival and that of CD34-positive neovascularization for incidence of esophageal varices. They have prepared quite large number of

patients for their retrospective study regarding the incidence of BA. The results seem quite reasonable, especially the positive relationship between hepatic neovascularization and incidence of esophageal varices is novel although the relationship between liver fibrogenesis and clinical outcome of PE has already widely known as well as that between age performed PE and liver fibrogenesis.

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Protective effects of intravenous anesthetics on kidney tissue in obstructive jaundice

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Abstract

AIM: To evaluate the protective effects on kidney tissue of frequently used intravenous anesthetics (ketamine, propofol, thiopental, and fentanyl) in rats with obstructive jaundice.

METHODS: There is an increased incidence of postoperative acute renal failure in patients with obstructive

jaundice. Thirty-two Wistar-albino rats were randomly divided into four equal groups. Laparotomy was performed on each animal in the four groups and common bile ducts were ligated and severed on day 0. After 7 d, laparotomy was again performed using ketamine, propofol, thiopental, or fentanyl anesthesia whose antioxidative properties are well known in oxidative stress in a rat liver model of obstructive jaundice. After 2 h, the rats were sacrificed. Renal tissue specimens were analyzed for catalase, superoxide dismutase and malondialdehyde enzymes activities. All values are expressed as the mean \pm SD. *P* values less than 0.05 were considered statistically significant.

RESULTS: All animals survived without complications until the end of the study. Enlargement in the bile duct and obstructive jaundice were observed in all rats. Catalase was found to be significantly lower in the fentanyl group than in the ketamine ($P = 0.039$), propofol ($P = 0.012$), and thiopental ($P = 0.001$) groups. Superoxide dismutase activities were similar in all groups ($P > 0.05$). Malondialdehyde was found to be significantly lower in the ketamine group than in the propofol ($P = 0.028$), thiopental ($P = 0.002$) and fentanyl ($P = 0.005$) groups. Malondialdehyde was also lower in the fentanyl group than in the thiopental group ($P = 0.001$). The results showed that obstructive jaundice sensitizes renal tissue to damage under the different anesthetics.

CONCLUSION: Among the agents tested, ketamine and propofol generated the least amount of oxidative stress on renal tissues in this rat model of obstructive jaundice created by common bile duct ligation. The importance of free radical injury in renal tissue in obstructive jaundice under different intravenous anesthetics during hepatobiliary and liver transplant surgery should be considered for prevention of postoperative acute renal failure.

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Key words: Obstructive jaundice; Postoperative acute renal failure; Oxidative stress; Intravenous anesthetics; Renal tissue damage

Core tip: There is an increased incidence of postoperative acute renal failure in patients with obstructive jaundice. Recent studies suggested that the free oxygen radicals produced in obstructive jaundice may play a major role in the etiopathogenesis of acute renal failure. We evaluated the protective effects on kidney tissue of frequently used intravenous anesthetics, whose antioxidative properties are well known, in a rat model of obstructive jaundice. Among the agents tested, ketamine and propofol generated the least amount of oxidative stress on renal tissues in this rat model of obstructive jaundice created by common bile duct ligation.

Hatipoglu S, Yildiz H, Bulbuloglu E, Coskuner I, Kurutas EB, Hatipoglu F, Ciralik H, Berhuni MS. Protective effects of intravenous anesthetics on kidney tissue in obstructive jaundice. *World J Gastroenterol* 2014; 20(12): 3320-3326 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i12/3320.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i12.3320>

INTRODUCTION

Today, palliative and curative operations are performed on many patients with obstructive jaundice (OJ) under anesthesia. As a result of improvements in liver transplant surgery in the last 50 years, more complicated and prolonged operations are being conducted. Patients with severe OJ usually have a number of metabolic disorders and one or multiple organ function failure. Renal dysfunction is one of the serious complications in patients with OJ^[1-3].

An association between OJ and acute renal failure (ARF) has been recognized for well over a century. The renal damage is due to biliary disorders either present on admission to hospital or which develop postoperatively. One third of patients with OJ have deterioration of renal function before surgical intervention^[4]. On the other hand, surgery on patients with OJ is known to be associated with increased risk of postoperative renal failure^[5-8]. Early diagnosis and prevention of spontaneous evolution of the disease can improve prognosis.

Patients with intra- or extra-hepatic bile duct obstruction are susceptible to ARF especially after major surgery^[9]. Surgical treatment for the relief of OJ is still complicated by postoperative ARF in almost 10% of patients^[3]. Patients with OJ are often subjected to either general or sedation anesthesia, usually using drugs which are metabolized by the liver and/or are eliminated by the kidney and the liver. Some intravenous anesthetic agents have been shown to increase production of reactive oxygen species and cause tissue damage^[9-13]. In contrast, some intravenous anesthetic drugs are capable of reduc-

ing oxidative stress^[13,14].

To date, there have been no reports of the effects of intravenous anesthetic agents on oxidative stress in renal tissues in rats with OJ. Biliary obstruction is associated with an intense state of oxidative stress. Antioxidant defenses [as demonstrated by superoxide dismutase (SOD) and catalase (CAT) activities] are decreased and lipid peroxidation [as demonstrated by malondialdehyde (MDA) levels] are increased in rat models with OJ^[13,15]. In this study, we therefore investigated the effects on renal tissues of frequently used intravenous anesthetics (ketamine, propofol, thiopental, and fentanyl), in a rat model of oxidative stress caused by OJ through common bile duct ligation. We used these intravenous anesthetics as their antioxidative properties are well known.

MATERIALS AND METHODS

Animals

The experimental protocol was approved by the Animal Ethics Review Committee of the Faculty of Medicine, University of Kahramanmaraş and adhered to the National Institutes of Health Guidelines for the Use of Experimental Animals. Thirty-two male Wistar rats (300-375 g) were subjected to controlled conditions of temperature (about 22 °C) and illumination (12 h light:12 h dark cycle), and were provided with food and water *ad libitum*. They were fed a commercial diet. Rats were placed in individual metabolic cages and acclimatized for 1 wk before the study commenced.

Experimental design

In this prospective experimental study, rats were divided randomly into four groups, each group containing eight animals. Food was withdrawn for 12 h before the operation, with water available *ad libitum* during this period. Each rat was weighed during each anesthetic and anesthetized with ketamine (50 mg/kg) intramuscularly. As described by Lee *et al.*^[16], experimental jaundice was created by ligation of the common bile duct^[13,15]. The abdominal cavity was opened with a midline incision after disinfection of the skin. The common bile duct was located and OJ induced by double ligation with 5/0 silk and transection of the common bile duct in the supraduodenal part between the lowermost tributary of the bile duct and the uppermost tributary of the pancreatic duct. The abdominal wall was then closed with 3-0 silk in two layers. Cages were examined daily.

After 7 d, Group I rats received intramuscular single-dose ketamine (50 mg/kg), Group II received intramuscular single-dose propofol (10 mg/kg), Group III received intramuscular single-dose thiopental (20 mg/kg), and Group IV received intramuscular single-dose fentanyl (50 mcg/kg). Two hours later, the rats were sacrificed.

Sample collection

The animals were anesthetized and a second laparotomy was performed through a similar incision. The left and

Table 1 Mean malondialdehyde, superoxide dismutase levels and catalase levels in renal tissue of rats

Groups	Catalase	Superoxide dismutase	Malondialdehyde
B1 (ketamine)	146.11 ± 20.91 ¹	3.52 ± 0.73	0.27 ± 0.080 ^{4,5,6}
B2 (propofol)	154.11 ± 21.46 ²	3.74 ± 0.67	0.50 ± 0.24 ⁴
B3 (thiopental)	174.8 ± 36.6 ³	3.70 ± 0.61	0.73 ± 0.22 ^{5,7}
B4 (fentanyl)	122.48 ± 20.54 ^{1,2,3}	3.41 ± 0.59	0.47 ± 0.19 ^{6,7}

Data are expressed as mean ± SD. Mean malondialdehyde (MDA), superoxide dismutase (SOD) levels and catalase (CAT) levels in renal tissue of rats (8 rats in each group). CAT and SOD activities are expressed as U/mg protein. MDA enzyme activities are expressed as nmol/mg protein. *P*-values < 0.05 were considered statistically significant. In the CAT group: ¹*P* = 0.039 for ketamine *vs* fentanyl comparison; ²*P* = 0.012 for propofol *vs* fentanyl comparison; ³*P* = 0.001 for thiopental *vs* fentanyl comparison. In the MDA group: ⁴*P* = 0.028 for ketamine *vs* propofol comparison; ⁵*P* = 0.002 for ketamine *vs* thiopental comparison; ⁶*P* = 0.005 for ketamine *vs* fentanyl comparison; ⁷*P* = 0.001 for thiopental *vs* fentanyl comparison.

right kidneys of each rat were carefully removed in all groups and stored in iced 0.9% NaCl solution for a short time. A 0.5 cm × 0.5 cm sample of kidneys (left or right) which contain both renal cortical and medullar tissue were washed with physiological saline to remove hematoma and blotted on filter paper. The renal tissue was immediately frozen in liquid nitrogen and stored at -80 °C for later measurement of MDA, SOD and CAT activities.

Antioxidant study

In order to determine tissue antioxidant levels, the renal tissue samples were removed from the freezer, brought to room temperature, then homogenized with three volumes of ice-cold 1.15% KCl. Activities of antioxidant enzymes and levels of lipid peroxidation were measured in the supernatant after centrifugation at 14000 rpm. SOD activity was measured by the method described by Fridovich^[17]. CAT activity was determined by measuring the decrease in hydrogen peroxide concentration at 230 nm by the method of Beutler^[18]. Lipid peroxidation was reflected by MDA levels, which were measured by the method described by Ohkawa *et al.*^[19]. All enzyme activities are expressed as units per milligram protein (U/mg protein).

Statistical analysis

All values are expressed as mean ± SD. The Kolmogorov-Smirnov statistic was used to test the normality of the distribution. Differences between SOD groups were evaluated by Kruskal-Wallis variance analysis followed by a *post hoc* (Bonferroni correction) Mann-Whitney *U* test. Differences between MDA and CAT groups were evaluated by one way analysis of variance (ANOVA) for continuous variables with *post hoc* procedures (Bonferroni correction). *P*-values < 0.05 were considered statistically significant. Data were analyzed using SPSS 9.05 for Windows® statistical package (Chicago, IL, United States).

RESULTS

All animals survived without complications until the end of the study. Enlargement of the bile duct and OJ were observed in all rats. The mean values of the parameters studied are presented in Table 1. The results showed that the presence of OJ sensitized the renal tissue to damage under the different anesthetics.

CAT was found to be significantly lower in the fentanyl group than in the ketamine (*P* = 0.039), propofol (*P* = 0.012), and thiopental (*P* = 0.001) groups. Although CAT was higher in the thiopental group than in the ketamine and propofol groups, this difference was not statistically significant (Table 1, Figure 1A).

SOD activity was similar between all groups and intergroup differences were not found (*P* > 0.05) (Table 1, Figure 1B).

MDA was found to be significantly lower in the ketamine group than in the propofol (*P* = 0.028), thiopental (*P* = 0.002) and fentanyl (*P* = 0.005) groups. MDA was also lower in the fentanyl group than in the thiopental group (*P* = 0.001). MDA was similar between the propofol and thiopental groups and no other significant intergroup difference was found (Table 1, Figure 1C).

DISCUSSION

Many clinical observations and experimental studies point to the frequent occurrence of different organ complications in patients with OJ. One of the main consequences of biliary obstruction is its effect on renal function, which markedly increases patient morbidity and mortality. Acute renal failure is a life-threatening complication of OJ which continues to be a significant challenge, involving 6%-18% of patients, and is associated with high mortality (20%-100%)^[20-23]. Patients with intra- or extra-hepatic bile duct obstruction are susceptible to ARF especially when undergoing major surgery, and postoperative ARF in patients with OJ remains a clinically significant complication^[9,24]. ARF occurs in approximately 9% of patients requires surgery for relief of OJ, and contributes to eventual mortality in 76% of those who develop it. Postoperative mortality has been directly attributed to ARF in approximately 5%-16% of patients after surgery for OJ^[25,26].

When mechanical biliary obstruction is diagnosed, surgical, endoscopic or radiologic intervention is usually recommended. On the other hand, despite advances in preoperative evaluation and postoperative care, surgical intervention for relief of obstructive jaundice still carries high morbidity and mortality rates, mainly due to sepsis and renal dysfunction^[25,27-30]. The presence of OJ (total bilirubin < 8 mg/dL) is an independent risk factor for the development of postoperative renal dysfunction^[31].

Although the association between OJ and ARF has been recognized since 1910 when Clairmont and Von Haberer^[22] first postulated that jaundice might predispose to postoperative renal failure, surprisingly few reports or

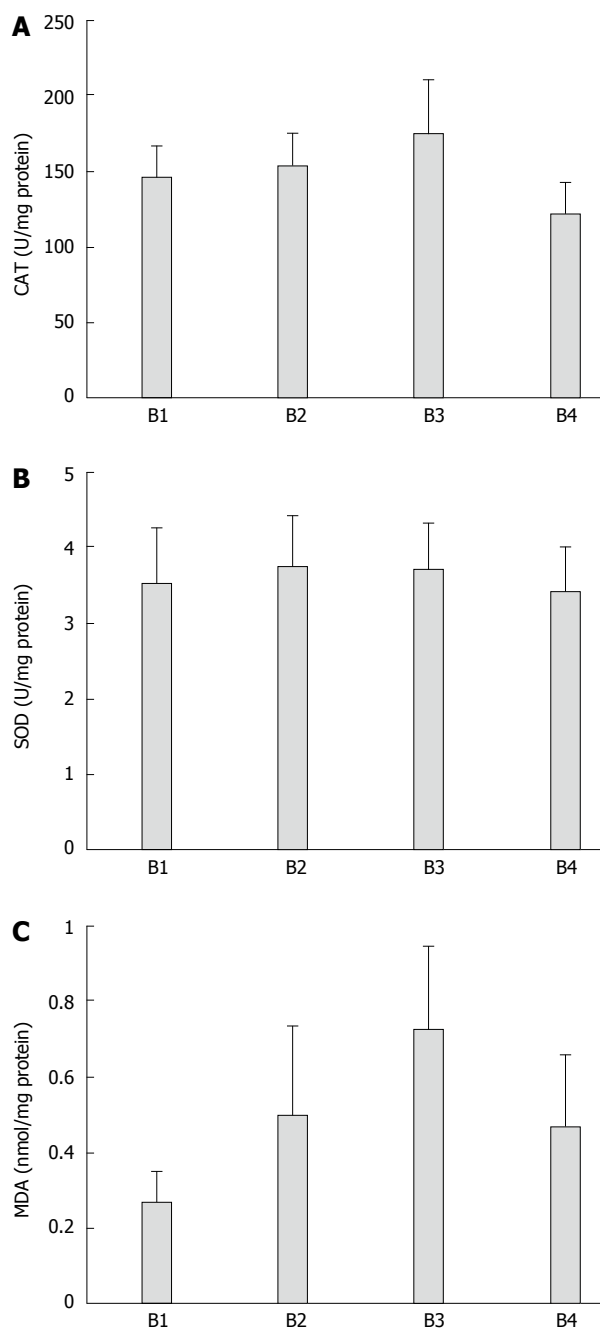


Figure 1 Mean malondialdehyde, superoxide dismutase levels and catalase activity in our groups [B1 (ketamine), B2 (propofol), B3 (thiopental) and B4 (fentanyl)]. A: Catalase (CAT); B: Superoxide dismutase (SOD); C: Malondialdehyde (MDA).

series have appeared in the literature^[20,32]. Antifibrinolytic agents, OJ, prostaglandin inhibitors, cyclosporine A, radiocontrast dyes and volatile anesthetic agents contribute to ARF^[31,33,34]. There is a lack of knowledge about the oxidative effects of intravenous anesthetic agents in renal tissue during surgery for OJ patients.

Postoperative ARF may be precipitated in patients with OJ either by surgery or septicemia or a combination of both. Effective plasma volume depletion, systemic endotoxemia, and myocardial dysfunction contribute to hemodynamic and renal disturbance in patients with

acute OJ^[32]. Intrarenal vasoconstriction, attributable to a decrease in effective arterial blood volume and induced by peripheral arterial vasodilation, is proposed to play a causative role in OJ^[35]. Decreased creatinine clearance and urine osmolality are the parameters which point to the probability of renal dysfunction occurrence in OJ^[4]. Although the etiology of renal dysfunction is multifactorial, it is strongly associated with hemodynamic and body fluid disturbances. However, the etiology of this clinical status is still unclear.

Oxidative stress occurs during many pathological processes in an organism. Free oxygen radicals are neutralized by the antioxidant system and a balance is maintained. A major protective mechanism against reactive oxygen metabolites is also the antioxidant enzyme cascade^[36-38]. Antioxidant defenses (as demonstrated by CAT and SOD activities) are decreased and lipid peroxidation (as demonstrated by MDA levels) are increased during extrahepatic OJ in rat models^[13,15]. When this balance is impaired, however, tissue damage may result. Oxidant injury is considered to be an important mechanism in the pathophysiology of ARF^[36-40] and severe oxidative stress has been implicated in the renal dysfunction associated with experimental OJ^[41]. Ischemia and nephrotoxicity are important factors in the pathogenesis of ARF and their effects on renal cells can be loss of SOD and superoxide radical accumulation^[36]. Oxygen free radicals produce damage to the renal arteriolar endothelial cells, glomerular cells, and renal tubular epithelial cells^[36-38].

Renal tissues in OJ appear to be susceptible to ischemia-reperfusion injury^[42]. Tissue injury induced by OJ involves lipid peroxidation^[43]. Experimental animals with OJ have been shown to be deficient in fat-soluble vitamins, such as vitamins A and E^[13,43]. Because these vitamins have potential to ameliorate secondary tissue damage induced by lipid peroxidation, enhanced oxidative stress could exacerbate secondary tissue damage. Moreover, OJ could alter the activities of antioxidant enzymes resulting in increased production of superoxide and hydrogen peroxide^[44]. Tissue damage associated with OJ may be caused by accelerated generation of hydroxyl radicals^[43-46]. Oxidative stress seems to be a cardinal feature of cholestasis, implicated in the pathophysiology of organ injury not only in the liver, but also in renal tissues^[47]. Superoxide radicals may play an important role in the pathophysiology of cholestatic liver injury, intestinal barrier failure and ARF^[47].

Commonly used intravenous agents have been shown to increase oxygen production and generate tissue damage^[9-13,48]. Intravenous anesthetic agents generate free radicals by altering intracellular cytochrome p450, peroxisomes, and enzymatic systems in the mitochondria^[9]. Moreover, they consume and inhibit enzymatic and non-enzymatic systems that protect the cells *via* scavenging free radicals. They cause lipid peroxidation, DNA damage and changes in proteins by inducing oxidative damage, which eventually may lead to alterations in cellular functions such as reduced gap junction-mediated trans-

mission, activation of transcription factors, intracellular calcium and pH changes, and/or cell death^[9-13,49].

Transient functional impairment of renal cation and water transport in the proximal convoluted tubule occurred 3 to 4 d following bile duct ligation in rats^[6]. Maximum plasma concentrations and renal clearance of bile acids occurred between the 3rd or 4th postoperative day following common bile duct ligation. This peak coincided with maximal disruption of the proximal convoluted tubule architecture and postoperative changes in renal function such as increased urine flow rate and decreased urine osmolality and sodium excretion^[6]. Because of these results, we chose to sacrifice our rats on the 7th postoperative day and specimens of renal tissues were resected.

Ketamine has been extensively studied as a safe and reliable dissociative sedative/anesthetic agent in various clinical situations. Ketamine's properties as a protective agent against oxidative stress and ischemia/reperfusion injury of the brain, kidney, skeletal muscle, heart, and intestine have been reported^[50-54]. In our experiment, MDA levels were lower in the ketamine group compared with the other groups, confirming ketamine as an agent which protects against oxidative stress. MDA is one of the fairly reactive metabolic products resulting from the effect of free oxygen radicals on tissues and from a series of reactions during lipid peroxidation. The tissue MDA level is a sensitive indicator of lipid peroxidation and thus of oxidative stress^[55]. Since ketamine lowered MDA levels more than the other agents used, we can conclude that it has an influence over the antioxidant defense system, while reducing lipid peroxidation.

Propofol and thiopental are another type of highly lipid-soluble anesthetic which have demonstrated antioxidant properties by inhibiting lipid peroxidation^[56-58]. Both are often used to reduce cerebral edema during liver transplantation in fulminant hepatic failure patients^[13]. Propofol is widely used for the induction and maintenance of general anesthesia, as well as for sedation of intubated postoperative patients on mechanical ventilation. Propofol has been proven to ameliorate ischemic/reperfusion injury in several organs, including the heart^[59], lungs^[60], brain^[61], and kidney^[62]. Propofol has been found to limit oxidative injury in the liver and other tissues^[63]. According to our literature searches, the oxidative effects of propofol and thiopental on renal tissue injury due to OJ have not been studied before now. Regarding the markers of oxidative stress, MDA was highest in the thiopental group, and was significantly higher than in the ketamine or fentanyl groups. Although CAT was higher in the thiopental group than in the ketamine and propofol groups, this difference was not statistically significant. SOD catalyzes the produced superoxide radicals into H₂O₂, whereas CAT prevents oxidative damage by dissociating H₂O₂ and inhibiting lipid peroxidation^[64]. In our experiment, SOD activity was similar amongst all groups and no significant intergroup difference was found.

Fentanyl is one of many opioid receptor agonists and has effects on the brain, heart, and liver^[65]. Regarding its

oxidative effects on renal tissue in OJ however, little is known. In our experiment, CAT was found to be significantly lower in the fentanyl group than in the ketamine, propofol, and thiopental groups.

The association between ARF and OJ is well established. However, despite the substantial number of clinical reviews, animal studies, and various pathogenic mechanisms and therapeutic strategies proposed, the main pathophysiological mechanisms are still obscure. Therefore, postoperative ARF remains a major challenge in hepatobiliary and liver transplant surgery. It is important to recognize ARF early and take adequate measures to prevent its occurrence. As free oxygen radicals appear to play a significant role in ARF etiopathogenesis, one option is preoperative and postoperative antioxidant treatment to prevent ARF in OJ. According to our experiment, ketamine and propofol generated the least amount of oxidative stress in renal tissues in this rat model of OJ created by common bile duct ligation. In addition, close collaboration of clinicians, especially hepatobiliary and liver transplant surgeons and anesthesiologists, is very important during the preoperative, perioperative, and postoperative process to prevent ARF.

COMMENTS

Background

The association between acute renal failure and obstructive jaundice is well established and there is an increased incidence of postoperative acute renal failure in patients with obstructive jaundice. Recent studies suggest that the free oxygen radicals produced in obstructive jaundice may play a major role in the etiopathogenesis of acute renal failure. The authors evaluated the protective effects on kidney tissue of frequently used intravenous anesthetics whose antioxidative properties are well known in oxidative stress in a rat liver model of obstructive jaundice.

Research frontiers

The importance of free radical injury on renal tissue in obstructive jaundice under different intravenous anesthetics should be considered during hepatobiliary surgery for prevention of postoperative acute renal failure.

Innovations and breakthroughs

To date, no one has reported the effects on renal tissues of intravenous anesthetic agents on oxidative stress in a rat model of obstructive jaundice. Biliary obstruction is associated with an intense state of oxidative stress. Antioxidant defenses (as demonstrated by superoxide dismutase and catalase activities) are decreased and lipid peroxidation (as demonstrated by malondialdehyde levels) are increased in rat models with obstructive jaundice. Ketamine and propofol generated the least amount of oxidative stress in renal tissues in this rat model of obstructive jaundice created by common bile duct ligation.

Peer review

The paper describes how different anesthetics could potentially reduce the risk of acute renal failure in patients with obstructive jaundice by reducing the oxidative stress inflicted by jaundice in combination with acute surgery. So that, it is of interest and should be of interest to the readers.

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Thiopurine-methyltransferase variants in inflammatory bowel disease: Prevalence and toxicity in Brazilian patients

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with drug toxicity in inflammatory bowel disease (IBD) patients from southeastern Brazil.

METHODS: A total of 219 consecutive patients with IBD, of which 146 had Crohn's disease and 73 had ulcerative colitis, regularly seen at the outpatient unit of the Division of Gastroenterology at the University Hospital Pedro Ernesto of the State University of Rio de Janeiro, a tertiary referral center, were enrolled in this study from February 2009 to January 2011. We analyzed the presence of major TPMT genetic variants (TPMT*2, *3A, *3C) in IBD patients by means of a specific allele and RFLP-PCR. Genomic DNA was isolated from peripheral blood leukocytes by proteinase-K/Sodium Dodecyl Sulfate digestion and phenol-chloroform extraction. TPMT*2 (C238G), TPMT*3A (G460A/A719G), and TPMT*3C (A719G) genotypes were detected by real-time polymerase chain reaction followed by direct sequencing with specific primers. Clinical data were systematically recorded, and correlated with the genotype results.

RESULTS: The distribution of the selected *TPMT* gene polymorphism TPMT*2 (C238G), TPMT*3A (G460A/A719G), and TPMT*3C (A719G) genotypes was 3.6%, 5.4%, and 7.7% of the patients, respectively. Among the side effects recorded from patients taking azathioprine, 14 patients presented with pancreatitis and/or an elevation of pancreatic enzymes, while 6 patients had liver toxicity, and 2 patients exhibited myelosuppression/neutropenia. TPMT polymorphisms were detected in 37/219 patients (8 heterozygous for *2, 11 heterozygous for *3A, and 18 heterozygous for *3C). No homozygous polymorphisms were found. Despite the prevalence of the TPMT*3C genotype, no differences among the genotype frequencies were significant. Although no association was detected regarding myelotoxicity or hepatotoxicity, a trend towards the elevation of pancreatic enzymes was observed for TPMT*2 and TPMT*3C genotypes.

Abstract

AIM: To analyze the prevalence of thiopurine-methyltransferase (TPMT) genotypes and their association

CONCLUSION: The prevalence of TPMT genotypes was high among Brazilian patients. Variants genes *2 and *3C may be associated with azathioprine pancreatic toxicity in a IBD southeastern Brazilian population.

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Key words: Inflammatory bowel disease; Thiopurine-methyl-transferase; Azathioprine; Drug toxicity; Pancreatitis

Core tip: Although commonly used to treat patients with inflammatory bowel disease, the potentially severe side effects of azathioprine remain a concern. To determine a patient's predisposition to azathioprine toxicity, the thiopurine-methyl-transferase genotype must be determined prior to azathioprine administration.

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INTRODUCTION

The purine analogs azathioprine (AZA) and 6-mercaptopurine (6-MP) are the most common immunosuppressant drugs used to treat inflammatory bowel disease (IBD) and have been shown to be effective in inducing and maintaining remission^[1-5]. Recent evidence has indicated that azathioprine can control active inflammation and prevent relapse in Crohn's disease (CD) and ulcerative colitis (UC), as well as reduce steroid dependence and induce and maintain remission in CD. Many of these beneficial effects have been attributed to the ability of AZA to induce T cell apoptosis^[6,7]. However, in one third of patients, treatment with AZA is withdrawn due to either toxicity (gastrointestinal intolerance, pancreatitis, and bone marrow suppression in 9%-28% of patients) or a lack of a clinical response (15% of cases)^[6,8].

The metabolism of AZA and 6-MP is complex. AZA is a pro-drug, which is absorbed into the plasma and rapidly converted to 6-MP *via* a glutathione-dependent process. 6-MP can be inactivated by xanthine oxidase (XO) and thiopurine-methyl-transferase (TPMT) or converted to cytotoxic 6-thioguanine nucleotides (6-TGNs) *via* a multi-enzymatic process^[7,9,10]. TPMT or XO deficiency results in greater conversion of 6-MP to 6-TGNs, which are the predominant active metabolites related to drug efficacy, but this conversion also induces toxicity. Patients with low TPMT activity display higher 6-TGN levels when treated with standard doses of AZA and are at in-

creased risk of myelosuppression. However, patients with high TPMT activity are usually resistant to thiopurines or require a higher dose to achieve efficacy, which increases the risk of hepatotoxicity^[7,9-12].

However, the large variability in the activity of TPMT among patients has been attributed to TPMT gene polymorphisms^[13,14]. The TPMT gene has been localized to chromosome 6p22.3. A total of 24 TPMT genetic polymorphisms have been identified; these polymorphisms are associated with decreased levels of enzymatic activity and/or thiopurine drug-induced toxicity. These activities are genetically determined and demonstrate a trimodal distribution in the Caucasian population: 88.6% of individuals carry two allele types resulting in normal or high TPMT activity (TPMT *1), 0.3% are homozygous for low activity alleles with no detectable TPMT activity, and 11.1% are heterozygous and display intermediate activity^[13,14]. The overall concordance rate between TPMT genetic polymorphisms and specific phenotypes is 98.4%^[7,9,10,12,15].

Three mutant alleles, TPMT *2, *3A, and *3C, account for the majority of the variant alleles in the entire human population studied to date. Intermediate or low TPMT activity is most frequently associated with these allele polymorphisms^[13,14]. The distribution of these alleles differs significantly among ethnic populations, particularly the *3A and *3C genotypes^[7,9,11,12]. However, a number of studies have shown that the frequency of these alleles does not differ in IBD patients compared to controls within the same populations.

The aim of this study was to determine the prevalence of TPMT gene polymorphisms in a group of Brazilian patients with IBD and to investigate the relationship between these polymorphisms and thiopurine related-toxicity and treatment response.

MATERIALS AND METHODS

Study population

A total of 219 consecutive patients with IBD, of which 146 had CD and 73 had UC, regularly seen at the outpatient unit of the Division of Gastroenterology and Gastrointestinal Endoscopy at the University Hospital Pedro Ernesto of the State University of Rio de Janeiro, a tertiary referral center, were enrolled in this study from February 2009 to January 2011. The diagnosis of IBD was based on established diagnostic criteria, including clinical, imaging, endoscopic, and histological parameters.

The following data were collected from all patients: gender, age, age at diagnosis, disease activity, history of IBD-related surgery, chronic steroid use including steroid-dependent or steroid-refractory disease, and the presence of side effects from medical treatment. For patients with CD, the disease location was characterized as the terminal ileum (L1), colon (L2), ileocolon (L3), or upper gastrointestinal tract (L4). In addition, the predominant disease behavior was defined as non-stricturing/non-penetrating (B1), stricturing (B2), and perforating (B3), according to

Table 1 Baseline demographic and clinical characteristics of the inflammatory bowel disease patients *n* (%)

Diagnosis	CD (<i>n</i> = 146)	UC (<i>n</i> = 73)
Gender		
Male:female	61:85	30:43
Age at diagnosis		
< 40 (A1):40 (A2)	111:35	38:35
Disease location CD		
Terminal ileum (L1)	30 (20.5)	
Colon (L2)	42 (28.8)	
Ileocolon (L3)	65 (44.5)	
Upper GI (L4)	9 (6.2)	
Disease behavior		
NS/NP (B1)	53 (36.3)	
Stricturing (B2)	45 (30.8)	
Penetrating (B3)	48 (32.9)	
Perianal disease		
Yes:no	38:108 (26.1)	
Disease location UC		
Pancolitis		38 (52.1)
Non-pancolitis		35 (47.9)
Disease activity		
Moderate/severe	28 (19.2)	15 (20.5)
Mild/Remission	118 (80.8)	58 (79.5)
Surgery because of IBD		
Yes:no	48:98 (32.9)	0:73 (0)
Chronic steroid use		
Yes:no	26:120 (17.8)	10:63 (13.7)
Side effects of medication		
Yes:no	43:103 (29.4)	18:55 (24.6)
Azathioprine		
Yes:no	138:8 (94.5)	44:29 (60.3)

CD: Crohn's disease; UC: Ulcerative colitis; IBD: Inflammatory bowel disease.

the Montreal classification^[16]. Perianal disease was considered separately as an additional feature. CD activity was based on the Crohn's Disease Index^[17]. For patients with UC, disease extension was based on the Montreal classification using a modified criteria that combined ulcerative proctitis and left-sided UC (E1 + E2) and included extensive UC (pancolitis; E3). Disease activity was assessed using the Truelove index^[18] (Table 1).

DNA extraction and genotyping

After a questionnaire containing clinical information was completed for each patient, peripheral blood samples were obtained from all participants by venipuncture and collection in EDTA tubes. Genomic DNA was isolated from peripheral blood leukocytes by proteinase-K/Sodium Dodecyl Sulfate digestion and phenol-chloroform extraction as previously described^[19]. The *TPMT* gene polymorphisms most commonly described in the literature, *TPMT**2 (C238G), *TPMT**3A (G460A/A719G), and *TPMT**3C (A719G) genotypes, were detected by real-time polymerase chain reaction (PCR) followed by direct sequencing with specific primers (Table 2) for each region of interest. These regions corresponded to exons 12, 21, and 26 of the *TPMT* gene. The genotype frequencies of the *TPMT* gene polymorphisms were analyzed in the study population of CD and UC patients. Genotype-phenotype associations with major clinical features were established,

Table 2 Primer sequences used to identify the G238C, G460A, and A719G polymorphisms of the thiopurine-methyl-transferase gene

Name	Sequence	Product size
P2W reverse	5' GTA TGA TTT TAT GACGGT TG 3'	254
P2M reverse	5' GTA TGA TTT TATGCA GGT TTC 3'	
P2C forward	5' TAA ATAGGAACC ATCGGA CAC 3'	
460 forward	5' TCC CCA AAT CAT AAC AGA GTG 3'	375
460 reverse	5' CTAGAACCCAGAAAAAGTATAG3'	
719 forward	5' CGT TGT CTT GAG AAG GTT GA 3'	175
719 reverse	5' CAT TAC ATT TTC AGG CTT TAG CAT A 3'	

and the estimated risks for the mutations were calculated.

Briefly, PCR was performed using a buffer containing 0.75 mmol/L MgCl₂, 0.2 mmol/L dNTPs, 1.0 U Platinum Taq DNA polymerase (all from Invitrogen, Life Technologies, Carlsbad, CA, United States), 20 pmol each primer, 200 ng genomic DNA, and sterile ultrapure water to a final volume of 50 µL. For amplification, the DNA was first denatured for 5 min at 94 °C, followed by 35 cycles of denaturation for 30 s at 92 °C, annealing for 30 s at 60 °C (exons 12 and 21) or 58 °C (exon 26), and extension for 1 minute at 72 °C. At the end of the 35 cycles, an additional 10-min cycle at 72 °C was performed. The PCR products were then purified with the Illustra GFX™ PCR DNA and Gel Band Purification kit according to the manufacturer's protocol (GE Healthcare, Buckinghamshire, United Kingdom).

The sequencing reactions were performed using the ET Dye Terminator Cycle Sequencing Kit (GE Healthcare, Buckinghamshire, United Kingdom) according to the manufacturer's protocol. The primers used were the same as those used for the PCR reaction (Table 2). For each product, eight sequencing reactions were performed; four contained sense oligonucleotides, and four contained antisense oligonucleotides.

PCR assay

To determine the G238C, G460A, and A719G polymorphisms, we performed PCR amplification in a final volume of 50 µL. The PCR reaction contained 100 ng genomic DNA, 1 U Platinum Taq polymerase, 1X reaction buffer, 10 mmol/L deoxynucleoside triphosphates (dNTP) (all from Life Technologies, United States) and primers (Table 2).

Detection of G238C

To detect the G238 polymorphism, a PCR assay was performed as previously described. Unpurified PCR products with a length of 256 base pairs were analyzed by agarose gel electrophoresis followed by staining with ethidium bromide. A DNA fragment was amplified with primers P2M and P2C when C238 (mutant) was present, whereas a DNA fragment was amplified with primers P2W and P2C when G238 (wild-type) was present (Figure 1).

Detection of G460A

To detect the G460A polymorphism, a PCR assay was

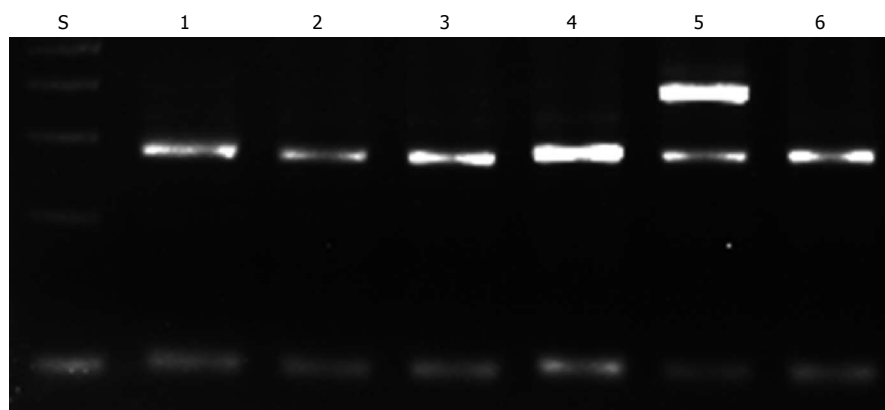


Figure 1 Agarose gel (2%) electrophoresis showing heterozygosis of thiopurine-methyltransferase*2 in one patients. S: Standard; 1, 2, 3, 4, 6: Wild homozygous; 5: Polymorphic heterozygous.

performed as previously described. The PCR product was digested with *Msp* I (New England Biolabs, Ipswich, MA, United States) for 1 h at 60 °C. The digested products were analyzed by gel electrophoresis. *Msp* I digestion of wild-type DNA yielded fragments of 267 and 98 base pairs, whereas DNA containing the G460A polymorphism was not digested, resulting in an uncleaved fragment of 365 base pairs.

Detection of A719G

To detect the A719G polymorphism, a PCR assay was performed as previously described. The PCR product was digested with *Acc* I (New England Biolabs, Ipswich, MA, United States) for 2 h at 37 °C and analyzed by electrophoresis. The A719G polymorphism generates an *Acc* I restriction site in the amplified fragment and yields fragments of 207 and 86 base pairs upon digestion. Wild-type DNA yielded an uncleaved fragment of 293 base pairs.

Statistical analysis

Tests for Hardy-Weinberg equilibrium were performed using the Genepop (Genepop web version 3.1) software. For all other data evaluations, we used SPSS 17.0 software (SPSS Inc., Chicago, IL, United States). The distribution of the individual characteristics was evaluated using simple descriptive statistics. Differences among the distributions of the selected variables were evaluated using either the chi-square test or the Fisher exact test for categorical data. All of the tests were two-tailed, and statistical significance was established at *P*-values of less than 0.05.

Ethical considerations

This study was approved by the Ethical Committee of the University Hospital Pedro Ernesto of the State University of Rio de Janeiro, and informed consent was obtained from all subjects (2310/2008). The study protocol was in accordance with the ethical principles for medical research involving human subjects statement of the Helsinki Declaration.

RESULTS

Genotype analysis

The prevalence of the selected *TPMT* gene polymorphism *TPMT**2 (C238G), *TPMT**3A (G460A/A719G), and *TPMT**3C (A719G) genotypes was 3.6%, 5.4%, and 7.7% of the patients, respectively. *TPMT* polymorphisms were detected in 37/219 patients, being 8 heterozygous for *2 (21.6%), 11 heterozygous for *3A (32.4%), and 18 heterozygous for *3C (46.0%). Despite the prevalence of the *TPMT**3C genotype, no differences among the genotype frequencies were significant. No homozygous polymorphism was found in this study. The allele frequencies in the study population are shown in Table 3. No significant difference was observed in the allele frequencies of the *TPMT* gene variants according to the predicted Hardy-Weinberg equilibrium.

Next, we investigated the prevalence of the *TPMT* gene variants and analyzed the distribution of each polymorphism with respect to the specific type of IBD (Table 4). For all three *TPMT* gene polymorphisms analyzed in this study, the frequencies of the wild-type (common homozygous), heterozygous, and homozygous polymorphic genotypes were similar among CD and UC patients (*P* = 0.722; *P* = 0.750; and *P* = 0.612, respectively). Importantly, no homozygous polymorphic types were observed.

Genotype-phenotype analysis

Next, we investigated the potential role of genotype in specific phenotypic variables. The associations between the sub-phenotypic categories and the genotype frequencies of the single nucleotide polymorphism (SNPs) in different subgroups of patients with CD and UC are shown in Table 5.

Among the side effects recorded from patients taking azathioprine, 14 patients presented with pancreatitis and/or an elevation of pancreatic enzymes, while 6 patients had liver toxicity, and 2 patients exhibited myelosuppression/neutropenia. No associations between the *TPMT**3A (G460A/A719G) polymorphism and specific CD or UC sub-phenotypes were observed. By contrast, we observed a trend towards a positive association be-

Table 3 Allelic frequencies of C238G, G460, and A719G in patients with inflammatory bowel disease

Polymorphism	CHz	HTz	RHz	n	Allelic frequency		χ^2	P-value
CD								
C238G	C:C	C:G	G:G		C	G		
Observed	141	5	0	146	0.98	0.02	0.04	0.83
Expected	141	5	0					
G460A/A719G	G:G/A:A	G:A/A:G	A/A:G/G		G/A	A/G		
Observed	137	9	0	146	0.97	0.03	0.14	0.70
Expected	137	9	0					
A719G	A:A	A:G	G:G		A	G		
Observed	128	18	0	146	0.94	0.06	0.63	0.42
Expected	128	16	1					
UC								
C238G	C:C	C:G	G:G		C	G		
Observed	71	2	0	73	0.99	0.01	0.01	0.90
Expected	71	2	0					
G460A/A719G	G:G/A:A	G:A/A:G	A/A:G/G		G/A	A/G		
Observed	70	3	0	73	0.98	0.02	0.03	0.85
Expected	70	3	0					
A719G	A:A	A:G	G:G		A	G		
Observed	66	7	0	73	0.95	0.05	0.18	0.66
Expected	66	7	0					

CD: Crohn's disease; UC: Ulcerative colitis; CHz: Common homozygous; HTz: Heterozygous; RHz: Rare homozygous. Data were analyzed using Fisher's exact test. If $P < 0.05$ -not consistent with HWE. Not accurate if < 5 individuals in any genotype group.

Table 4 Analysis of thiopurine-methyl-transferase gene polymorphisms in patients with inflammatory bowel disease *n* (%)

TPMT SNP	CHz	HTz	RHz	P-value
C238G	C:C	C:G	G:G	
CD (<i>n</i> = 143)	137 (95.8)	6 (4.2)	0	0.722
UC (<i>n</i> = 71)	69 (97.2)	2 (2.8)	0	
G460A/A719G	G:G/A:A	G:A/A:G	A/A:G/G	
CD (<i>n</i> = 126)	118 (93.7)	8 (6.3)	0	0.750
UC (<i>n</i> = 69)	66 (95.7)	3 (4.3)	0	
A719G	A:A	A:G	G:G	
CD (<i>n</i> = 135)	122 (90.4)	13 (9.6)	0	0.612
UC (<i>n</i> = 71)	66 (93.0)	5 (7.0)	0	

Data were analyzed using Fisher's exact test. CHz: Common homozygous; HTz: Heterozygous; RHz: Rare homozygous. CD: Crohn's disease; UC: Ulcerative colitis; TPMT: Thiopurine-methyl-transferase.

tween the TPMT*2 (C238G) and TPMT*3C (A719G) genotypes and the development of pancreatitis and/or abnormal levels of pancreatic-related enzymes in patients with IBD. For the TPMT*2 (C238G) genotype, amylase/lipase tended to be more elevated in patients with CD, while pancreatitis appeared to be more common in patients with UC. For the TPMT*3C (A719G) genotype, the levels of amylase/lipase tended to be higher among patients with CD. Other adverse effects, including myelosuppression/neutropenia and hepatotoxicity presented an overall low prevalence and were not associated with TPMT polymorphisms (Table 5).

DISCUSSION

This is the first study to investigate the TPMT*2, TPMT*3A and TPMT*3C genotypes in Brazilian patients with IBD. We determined that the prevalence of TPMT

gene polymorphisms is relatively high among Brazilian patients, including two genetic variants, TPMT*2 and TPMT*3C, that may be associated with pancreatic toxicity in IBD patients taking azathioprine. Nevertheless, the distributions of wild-type, heterozygous, and homozygous genotypes were similar among CD and UC patients, and no homozygous polymorphic type was observed.

Immunosuppressant agents such as Azathioprine and 6-MP are essential to promote and maintain remission in IBD patients. Unfortunately, major side effects have been associated with the use of these drugs, potentially limiting their utility. TPMT functions as a crucial enzyme in the metabolism of thiopurine drugs (including azathioprine and 6-MP) by methylating (*i.e.*, inactivating) these drugs^[10,20]. In IBD, genetic polymorphisms of TPMT have been associated with low or no enzyme activity, resulting in higher levels of metabolite concentrations and enhancing pharmacological activities and the risk of side effects^[21].

TPMT deficiency can also be measured by its activity in red blood cells or by genotypic determination. Although the methods are different, they yield similar results. Genotyping has greater clinical significance because it is not subject to external influences such as blood transfusions^[10,22,23]. TPMT gene polymorphisms can be easily identified, enabling the identification of patients with potential risk for drug toxicity. This is of particular interest in Brazil, which features a heterogeneous population and has been considered a low prevalence area for IBD, although the incidence has increased rapidly in the last decade^[15].

This study identified a TPMT gene polymorphism prevalence of 16.9% among patients with IBD, which is relatively high compared to reports in the literature for Caucasian populations (10% and 11%)^[7,20,21]. In other

Table 5 Association between the thiopurine-methyl-transferase polymorphisms and adverse effects in patients with inflammatory bowel disease *n* (%)

Adverse effect	C238G		G460A/A719G		A719G	
	CHz	HTz	CHz	HTz	CHz	HTz
Crohn's disease	35/143 (24.5)		30/126 (23.8)		33/135 (24.4)	
Myelosuppression	0	0	0	0	0	0
Neutropenia	3 (2.1)	0	2 (1.6)	0	2 (1.5)	0
Flu-like symptoms	3 (2.1)	0	3 (2.4)	0	3 (2.2)	0
Nausea/vomiting	12 (8.4)	0	9 (7.1)	1 (0.8)	11 (8.1)	1 (0.7)
Allergy/dermatitis	1 (0.7)	0	2 (1.6)	0	2 (1.5)	0
Hepatotoxicity	5 (3.5)	1 (0.7)	5 (4.0)	0	5 (3.7)	0
Amylase/lipase elevation	6 (4.2)	2 (1.4)	6 (4.7)	0	3 (2.2)	4 (2.9)
Pancreatitis	1 (0.7)	1 (0.7)	2 (1.6)	0	2 (1.5)	0
Ulcerative colitis	14/71 (19.7)		18/68 (26.5)		14/71 (19.7)	
Myelosuppression	0	0	0	0	0	0
Neutropenia	0	0	0	0	0	0
Flu-like symptoms	1 (1.4)	0	1 (1.5)	0	1 (1.4)	0
Nausea/vomiting	4 (5.6)	0	3 (4.4)	0	3 (4.2)	0
Allergy/dermatitis	3 (4.2)	2 (2.8)	3 (4.4)	0	2 (2.8)	0
Hepatotoxicity	0	0	0	0	0	0
Amylase/lipase elevation	2 (2.8)	1 (1.4)	3 (4.4)	0	3 (4.2)	0
Pancreatitis	0	1 (1.4)	1 (1.5)	0	1 (1.4)	0

CHz: Common homozygous; HTz: Heterozygous.

studies, the analysis of different populations has yielded more variable results, but the data on IBD patients did not differ from controls within the same populations. Studies in North American and European Caucasians indicate a predominance of the 3A genotype, which represents nearly 85% of all polymorphisms^[9,24]. Indjova *et al.*^[25] also observed a predominance of the 3A genotype in a healthy Bulgarian population, although with a lower prevalence (30.4%). In a cohort of ninety-seven thiopurine-treated paediatric IBD patients, 18 (18.56%) were heterozygous, while 2 (2.06%) were homozygous for a mutated *TPMT* gene^[26]. However, studies of African and Eastern Asian patients have indicated a predominance of the 3C genotype. In Asiatic studies, analyses of *TPMT* gene polymorphisms have detected only the 3C allele among a healthy Chinese population^[27] as well as among South Korean patients with IBD^[28]. By contrast, in western Asia, studies in Jordan^[15] and Iran^[29] detected different patterns of polymorphisms, and the occurrence of the 3C genotype was not exclusive.

In the present study, the distribution of genotypes among the 37 heterozygotes within the IBD population was 21.6% *TPMT**2, 32.4% *TPMT**3A, and 46% *TPMT**3C. With regard to the entire population in the study, this distribution corresponded to 3.6%, 5.4%, and 7.7% of the population, respectively, in contrast to previous studies of Caucasian (*3A) and Asian (*3C) populations, which usually report a clear predominance of a single gene polymorphism. Nevertheless, compared to other Brazilian data obtained from different regions and with a non-IBD population^[30-32], we observed an overall higher prevalence of *TPMT* gene polymorphisms. However, we observed a similar *3A and *3C allele distribution compared to other studies involving non-IBD patients from Rio de Janeiro^[30,32]. This finding is supported by the het-

erogeneous and highly mixed nature of the population in southeastern Brazil, particularly Rio de Janeiro, where European and African immigrants constitute a majority of the population background^[33].

Similar to other studies, we did not identify any significant association between *TPMT* gene polymorphisms and hepatotoxicity^[21] or myelotoxicity^[9,12]. It is possible that the sample size and/or the absence of polymorphic homozygotic patients may have influenced these results. However, interestingly, Gazouli *et al.*^[26] did not find any association between *TPMT* polymorphisms and the occurrence of thiopurine-related adverse events, even having detected a relatively high rate of polymorphic homozygotic among IBD patients. In this study, we observed a potential association of the *2 and *3C genotypes with pancreatic changes. An elevation of pancreatic enzymes was associated with the *TPMT**2 and *TPMT**3C genotypes in CD, while pancreatitis was more frequently observed in UC patients with the *TPMT**2 genotype. A possible association between specific *TPMT* genotypes and the pancreatic changes combined with the use of azathioprine in this study may support the dependence of these changes on genetic polymorphisms rather than idiosyncratic responses, as previously suggested^[21,34]. Thus, future studies with larger sample sizes and different populations will be necessary to confirm these findings.

In conclusion, our investigation of *TPMT* gene polymorphisms identified that the prevalence of *TPMT* gene polymorphisms is relatively high among Brazilian patients and a specific genetic profile among IBD patients from Rio de Janeiro. The possibility of specific *TPMT* genotypes be associated with adverse events, including potentially severe pancreatic toxicity, should alert physicians to the potential need to perform genetic testing prior to the initiation of therapy with thiopurine agents. Such

measures could facilitate the selection of appropriate medications and personalized doses of thiopurine agents to minimize toxic responses while maintaining full treatment efficacy.

COMMENTS

Background

The purine analogs azathioprine and 6-mercaptopurine (6-MP) are the most common immunosuppressant drugs used to treat inflammatory bowel disease (IBD). Thiopurine-methyl-transferase (TPMT) is a crucial enzyme in the metabolism of azathioprine and 6-MP. TPMT genetic polymorphisms are associated with decreased levels of enzymatic activity and/or thiopurine drug-induced toxicity.

Research frontiers

Evidence indicates the existence of TPMT activity differences between wild-type and heterozygous and homozygous mutated subjects. The carriers of at least one variant allele and with both intermediate and absent TPMT activity have an increased risk for developing thiopurine-induced myelotoxicity compared with individuals with normal genotype and TPMT activity. A high degree of concordance was demonstrated between TPMT genotype and phenotype.

Innovations and breakthroughs

In Caucasians, approximately 11% of the population harbour heterozygous and 0.3% homozygous TPMT mutations, leading to an intermediate or low TPMT activity, respectively. More than 24 mutations are now indexed but the clinical relevance of some of them remains unclear. TPMT*3A, TPMT*3C, and TPMT*2 represent the most prevalent mutant alleles in Caucasians and African-Americans and account for 80%-95% of intermediate or deficient methylator phenotypes.

Applications

In IBD patients from Rio de Janeiro the distribution of genotypes were predominantly TPMT*3A, and TPMT*3C, in contrast to previous studies of Caucasian (*3A) and Asian (*3C) populations, which usually report a clear predominance of a single gene polymorphism. This finding is supported by the heterogeneous and highly mixed nature of the population in southeastern Brazil, where European and African immigrants constitute a majority of the population background.

Terminology

Thiopurine-methyltransferase polymorphisms are relatively common among Brazilian patients with inflammatory bowel disease. In contrast to most studies, the rates of myelosuppression and hepatotoxicity were low, but a trend towards pancreatic toxicity may be associated with TPMT*2 and TPMT*3C genetic variants in IBD patients taking azathioprine.

Peer review

The authors firstly investigated the TPMT*2, TPMT*3A and TPMT*3C genotypes in Brazilian patients with IBD and demonstrated that the prevalence of TPMT gene polymorphisms is relatively high among Brazilian patients, including two genetic variants, TPMT*2 and TPMT*3C, that have been associated with pancreatic toxicity in IBD patients taking azathioprine. These results were interesting and important in clinical pretreatment of IBD.

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Propensity score analysis demonstrated the prognostic advantage of anatomical liver resection in hepatocellular carcinoma

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RESULTS: In the whole analysis set, the histological background of the liver, liver function, and tumor marker levels differed significantly among the groups. Although the overall survival (OS) and recurrence-free survival rates of the two groups did not differ significantly in the whole analysis set, the OS of the AR group was significantly longer than that of the NAR group after propensity matching (76.2 ± 6.3 mo vs 58.9 ± 6.3 mo; $P = 0.0039$). Although AR (HR = 0.456, $P = 0.039$) was found to be a prognostic factor in the univariate analysis, only vascular invasion (HR = 0.228, $P = 0.002$) and the hepatocyte growth factor level (HR = 52.366, $P = 0.035$) were subsequently found to be independent prognostic factors.

CONCLUSION: AR conveys a survival advantage over NAR in specific subpopulations of HCC patients with tumors of less than 5 cm in diameter, single tumor, and good liver function.

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Key words: Anatomical liver resection; Propensity score analysis; Hepatocellular carcinoma

Abstract

AIM: To compare the prognoses of hepatocellular carcinoma (HCC) patients that underwent anatomic liver resection (AR) or non-anatomic liver resection (NAR) using propensity score-matched populations.

METHODS: Between January 2002 and December 2010, 268 consecutive HCC patients, including 110 and 158 patients that underwent AR and NAR, respectively, were retrospectively enrolled in this study. Forty-four patients from each group were selected and matched using logistic multivariate analysis followed by propensity score analysis.

Core tip: The aim of this study was to compare the prognostic advantage of hepatocellular carcinoma (HCC) patients that underwent anatomic liver resection (AR) or non-anatomic liver resection (NAR) using propensity score-matched populations. Consecutive 268 HCC patients were enrolled and 44 patients from each group were matched using logistic multivariate analysis followed by propensity score analysis. The overall survival of the AR group was significantly longer than that of the NAR group after propensity matching. Vascular invasion and the hepatocyte growth factor level were subsequently found to be independent prognostic fac-

tors. AR conveys a survival advantage over NAR in specific subpopulations of HCC patients.

Ishii M, Mizuguchi T, Kawamoto M, Meguro M, Ota S, Nishida T, Okita K, Kimura Y, Hui TT, Hirata K. Propensity score analysis demonstrated the prognostic advantage of anatomical liver resection in hepatocellular carcinoma. *World J Gastroenterol* 2014; 20(12): 3335-3342 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i12/3335.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i12.3335>

INTRODUCTION

Liver resection is one of the curative approaches employed for hepatocellular carcinoma (HCC), which is the sixth most prevalent cancer worldwide^[1-3]. The optimal strategy for HCC management depends on the balance between the characteristics of the tumor and host liver function^[4-6]. Although the indications for liver resection for HCC recommend that only patients that retain good liver function should undergo the procedure^[7,8], the liver function of these patients can deteriorate due to chronic liver disease, including cirrhosis associated with viral hepatitis^[2,3].

The optimal type of liver resection for HCC has been debated and is divided into anatomic resection (AR) and non-anatomic resection (NAR)^[9-12]. Basically, AR is recommended for HCC patients who maintain good liver function^[11,13,14]. On the other hand, the clinical prognosis of cirrhotic patients that undergo NAR is comparable to that of cirrhotic patients that undergo AR^[15-17]. Meta-analysis of AR *vs* NAR has demonstrated the superiority of AR in specific subgroups^[18,19]. However, none of these reports were randomized control studies. Therefore, it is very difficult to compare the outcomes of the two surgical procedures due to the different tumor status and liver function backgrounds of the patients that undergo them, and so no conclusion about the matter has ever been reached.

To overcome the effects of patient background, performing multivariate analysis followed by propensity score-matched analysis makes it possible to compare elective groups whilst minimizing confounding factors in non-randomized retrospective studies^[20-22]. The aim of this study is to elucidate the prognostic differences among AR and NAR for HCC after matching gender, tumor characteristics, and liver function using propensity score analysis.

MATERIALS AND METHODS

Patients

Between January 2002 and December 2010, 268 consecutive HCC patients who underwent hepatectomy were recruited for this study after providing informed consent. Among the 268 patients, 110 underwent AR and 158

underwent NAR. The patients' tumors were evaluated by both ethoxybenzyl-enhanced magnetic resonance imaging and contrast-enhanced computed tomography scans were performed prior to surgery in order to assess tumor number and size. Clinical laboratory tests were carried out before surgery under stable conditions without inflammation. Histological evaluations of the tumor and liver parenchyma were carried out using surgical or biopsy specimens. Operative variables were recorded by the operating staff including the anesthesiologists. The design of this retrospective study conformed to the ethical guidelines of the Declaration of Helsinki, and all the patients gave their informed consent with individual signatures.

Surgical procedure

AR was defined as the complete removal of at least one Couinaud segment and exposure of the hepatic veins on the resected liver surface at the segment border. NAR was defined as the removal of the tumor regardless of the tumor margin or Couinaud segment without exposing the hepatic veins on the cut liver surface. All cases attempted to select AR, but some patients did not qualify for AR after liver resection due to unexposure of any segmental landmark or poor liver function. Indication of liver resection is based on same criteria^[23] during entire study period with same team. During surgery, a Cavitron ultrasonic sound aspirator and saline-linked electric cautery were used for the parenchymal dissection. When necessary, the pedicle of the hepatic hilum was intermittently clamped in cycles involving 10 min of clamping and 5 min of reperfusion.

Statistical analysis

For the statistical analyses, demographic data and perioperative laboratory test results were extracted from the clinical database, and the differences among the groups were compared using the χ^2 test followed by the post-hoc 2 \times 2 Fisher's exact test, when necessary. Continuous variables were compared using the Mann-Whitney *U* test. The factors affecting overall survival were assessed using the Kaplan-Meier method, with comparisons performed using the Log-rank test and univariate or multivariate analyses performed using the Cox proportional hazards regression model. Multivariate analyses were performed by backward selection of covariates with cut-off univariate *P* value of 0.05. To adjust for the different covariate distributions of the two groups (the AR and NAR groups), one-to-one matches were performed using propensity score analysis. The variables entered into the propensity model were gender, age, albumin level, bilirubin concentration, prothrombin time, tumor size, tumor number, operation time, and intraoperative blood loss. The model was then used to obtain one-to-one matches using the nearest-neighbor matching method. All calculations were performed using the StatView 5.0 software package (Abacus Concepts Inc., Berkeley, CA), NCSS (NCSS, Kaysville, UT), or SPSS 16.0 (SPSS Inc., Chicago,

Table 1 Clinicopathological characteristics of hepatocellular carcinoma patients who underwent initial hepatectomy in the full analysis set and one-to-one propensity score-matched pairs

Variables	Full analysis set			Propensity score-matched pairs		
	A (<i>n</i> = 110)	NA (<i>n</i> = 158)	<i>P</i> value	A (<i>n</i> = 44)	NA (<i>n</i> = 44)	<i>P</i> value
Gender (M:F)	97:13	133:25	0.455	38:6	38:6	NA
Age (yr)	68 (64-70)	66 (64-68)	0.862	64.9 ± 10.2	64.5 ± 9.5	0.838
Etiology (B:C:BC:NBNC)	50:32:1:27	70:61:6:21	0.035	19:18:1:6	24:12:1:7	0.602
Background (N:CH:L)	16:56:38	9:49:100	< 0.001	4:20:20	5:14:25	0.422
Histology (W:M:P)	50:32:1:27	29:94:35	0.138	6:30:8	8:28:8	0.836
Albumin (mg/dL)	3.91 ± 0.42	3.86 ± 0.47	0.321	3.99 ± 0.34	3.92 ± 0.41	0.379
Bilirubin (mg/dL)	0.6 (0.6-0.6)	0.8 (0.7-0.9)	< 0.001	0.68 ± 0.26	0.72 ± 0.39	0.545
PT (%)	93.3 ± 12.2	90.6 ± 13.6	0.106	92.4 ± 11.5	92.5 ± 12.4	0.948
ICGR15 (%)	8.5 (7.4-9.9)	13 (10.8-15)	< 0.001	10.4 ± 5.6	13.5 ± 8.8	0.053
Child-Pugh score (A:B)	109:1	153:5	0.419	44:0	44:0	NA
MELD score	7.68 ± 2.73	7.58 ± 1.39	0.674	7.78 ± 3.21	7.37 ± 1.29	0.448
Tumor size (cm)	4.2 (3.5-5.5)	2.5 (2.2-3.0)	< 0.001	3 (2.5-3.5)	3 (2.3-3.5)	0.904
No. of tumors	1 (1-1)	1 (1-1)	0.761	1 (1-1)	1 (1-1)	0.554
VI (-:+:++)	75:18:17	124:25:9	0.027	34:7:3	31:10:3	0.716
OT (min)	400 (364-439)	280 (260-300)	< 0.001	340.1 ± 105.8	322.7 ± 96.8	0.441
Blood loss (mL)	435 (380-600)	300 (230-390)	< 0.001	400 (310-482)	355 (270-560)	0.926
Blood transfusion (U)	1.3 ± 3.6	0.5 ± 2.3	0.049	0.4 ± 1.4	0.5 ± 1.6	0.833
BMI	23.19 ± 3.27	23.61 ± 3.38	0.313	23.57 ± 3.01	22.83 ± 3.13	0.263
Platelets	15.6 (13.3-17.1)	12.6 (11.7-14.2)	0.504	13.45 ± 4.71	16.41 ± 13.69	0.178
AST (IU/L)	36 (31-41)	36 (31-45)	0.206	40 (33-46)	33 (30-48)	0.439
ALT (IU/L)	34 (29-38)	32 (28-37)	0.413	38 (32-43)	29 (27-39)	0.103
MELD	7.03 (6.87-7.19)	7.12 (6.87-7.42)	0.674	7.10 (6.87-7.39)	7.03 (6.43-7.29)	0.396
BTR	6.07 (5.60-6.59)	5.42 (5.07-5.84)	< 0.001	5.89 (4.99-6.50)	5.73 (5.06-6.02)	0.277
Hyaluronate (ng/mL)	98 (76-132)	155 (128-196)	< 0.001	103 (66-139)	137 (104-187)	0.204
HGF (ng/mL)	0.32 (0.29-0.36)	0.36 (0.32-0.39)	0.069	0.33 ± 0.14	0.39 ± 0.15	0.066
AFP (ng/mL)	21.2 (11.2-80.9)	13.7 (8-29.5)	0.178	11.7 (6.5-38.4)	14.5 (5.8-46.4)	0.902
PIVKA (mAU/mL)	228 (90-639)	35 (27-53)	0.015	38 (24-158)	30 (23-66)	0.721

Median (95%CI of median) for skewed distribution and mean ± SD for normal distribution. A: Anatomical resection; NA: Non-anatomical resection; M: Male; F: Female; B: Hepatitis B; C: Hepatitis C; NBNC: Non-B and non-C hepatitis; N: Normal; CH: Chronic hepatitis; L: Liver cirrhosis; W: Well differentiated hepatocellular carcinoma; M: Moderately differentiated hepatocellular carcinoma; P: Poorly differentiated hepatocellular carcinoma; PT: Prothrombin time; ICGR15: Indocyanine green retention rate at 15 min; VI: Vascular invasion; OT: Operative time; BMI: Body mass index; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; MELD: Model for end-stage liver disease; BTR: Branched chain amino acids to tyrosine ratio; HGF: Hepatocyte growth factor; AFP: Alpha-fetoprotein; PIVKA: Protein induced by vitamin K absence or antagonist.

IL). All results are expressed as mean ± SD values. *P* values of < 0.05 were considered to be significant.

RESULTS

We retrospectively analyzed 268 HCC patients who initially underwent hepatectomy at our institute. Basically, AR was preferred, but some patients did not qualify for AR due to poor liver function and so were scheduled for NAR. In addition, no exposure of anatomical landmark after liver resection was also defined NAR. The full analysis set consisted of 110 and 158 patients that underwent AR and NAR, respectively (Table 1). After one-to-one matching using propensity score analysis, 44 pairs of patients were matched and compared. In the full analysis set, the histological background of the liver; indocyanine green retention rate at 15 min (ICGR₁₅); vascular invasion; operative time; intraoperative blood loss; blood transfusion volume; branched-chain amino acid to tyrosine ratio; and serum bilirubin, hyaluronate, hepatocyte growth factor (HGF), and protein induced by vitamin K absence levels differed significantly among the groups (*P* < 0.05). Among the propensity score-matched pairs,

none of these factors differed between the groups, indicating that the clinical backgrounds of the two groups had been successfully matched.

In the full analysis set, recurrence free survival (RFS) and overall survival (OS) did not differ significantly among the groups (Figure 1); *i.e.*, the median RFS of the AR group was 48.1 ± 5.2 mo, and that of the NAR group was 47.2 ± 4.8 mo (*P* = 0.282). In addition, the OS of the AR group was 94.5 ± 8.2 mo, and that of the NAR group was 78.2 ± 5.1 mo (*P* = 0.293).

On the other hand, among the propensity score-matched pairs the OS of the AR group was significantly longer than that of the NAR group (76.2 ± 6.3 mo *vs* 58.9 ± 6.3 mo, *P* = 0.0039) (Figure 2), although there was no significant inter-group difference in RFS (43.9 ± 7.1 mo *vs* 36.8 ± 5.8 mo, *P* = 0.213).

Multivariate analysis of the variables that were found to be significant predictors of OS in the univariate analysis revealed that although AR (HR = 0.456, *P* = 0.039), ICGR₁₅ (HR = 1.101, *P* < 0.001), tumor size (HR = 1.151, *P* = 0.001), vascular invasion (HR = 0.232, *P* < 0.001), blood loss (HR = 1.002, *P* = 0.001), serum aspartate transaminase level (HR = 1.024, *P* = 0.001), and serum HGF level

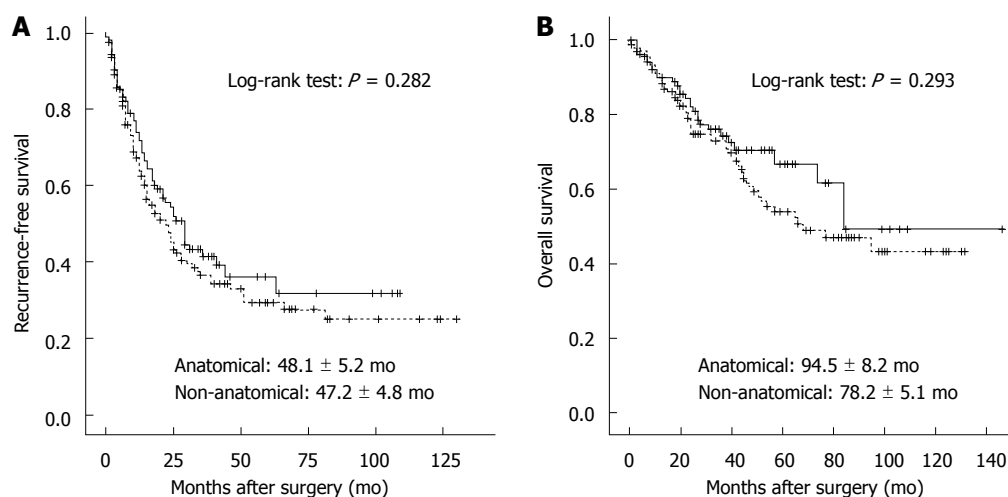


Figure 1 Recurrence-free survival (A) and overall survival (B) of hepatocellular carcinoma patients who underwent initial hepatectomy in the full analysis set. Anatomical resection (single line: $n = 110$); non-anatomical resection (dotted line: $n = 158$). $P < 0.05$ was considered to be significant.

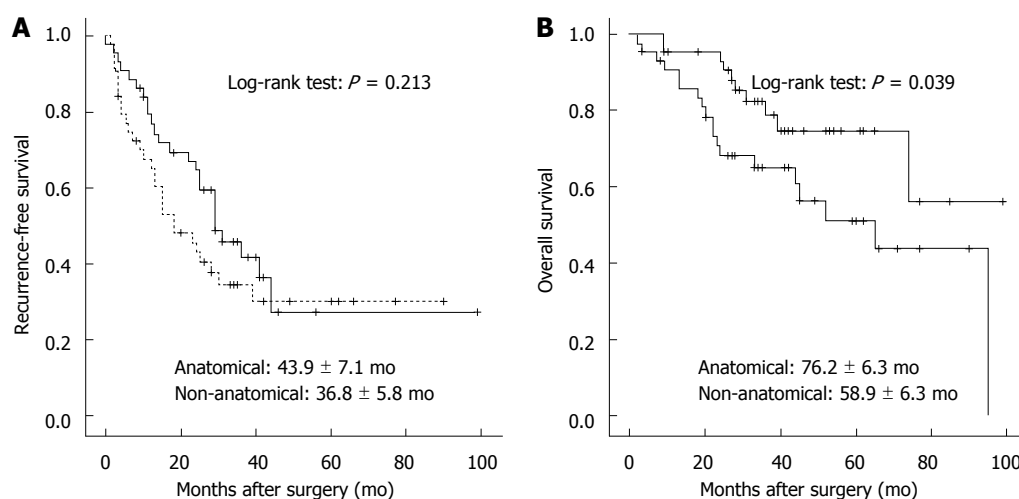


Figure 2 Recurrence-free survival (A) and overall survival (B) of hepatocellular carcinoma patients who underwent initial hepatectomy among the one-to-one propensity score-matched pairs. Anatomical resection (single line: $n = 44$); non-anatomical resection (dotted line: $n = 44$). $P < 0.05$ was considered to be significant.

(HR = 43.179, $P = 0.015$) were identified as prognostic factors in the univariate analysis, only vascular invasion (HR = 0.228, $P = 0.002$) and the HGF level (HR = 52.366, $P = 0.035$) were subsequently confirmed as independent prognostic factors in the multivariate analysis (Table 2).

DISCUSSION

We have demonstrated the survival benefit of AR compared with NAR for HCC patients who initially elect to undergo surgery, although the type of liver resection was not found to be an independent prognostic factor in the multivariate analysis. We have also demonstrated that propensity score-matched analysis can be used to compare specific therapies among selected subgroups.

AR for initial hepatectomy was selected for patients who possessed good liver function and reasonably sized tumors^[11,14,18,19,24]. Although the initial backgrounds of

the AR and NAR groups were significantly different, we successfully matched 44 patients from each group to produce pair with very similar clinical variables. In this matched patients, all cases attempted to select AR initially. AR is only qualified by the exposure of anatomical landmark, otherwise the others were defined as NAR. First of all, we need to examine the selection bias in this matched patient group. All of the matched patients belonged to Child-Pugh class A, had a mean tumor size of less than 5 cm, a mean tumor number of less than 1.5, and a mean BMI of less than 24. Therefore, our results were obtained under particular circumstances; *i.e.*, among lean patients with good liver function, tumors measuring less than 5 cm in diameter, and a small number of tumors (most patients only had one).

The prognosis of HCC patients after hepatectomy is determined by the balance between their liver function^[4,25,26] and the characteristics of their tumors, such

Table 2 Univariate and multivariate analysis of prognostic factors in the one-to-one propensity score-matched pairs

Overall survival Prognostic factors	Univariate analysis			Multivariate analysis		
	HR	95%CI	P value	HR	95%CI	P value
Anatomical resection	0.456	0.211-0.983	0.039	0.546	0.205-1.453	0.226
Female gender	0.673	0.157-2.877	0.572			
Age	0.994	0.955-1.033	0.747			
Background (N + CH)	0.867	0.412-1.825	0.707			
Histology (W + M)	0.442	0.194-1.005	0.068			
Albumin	0.536	0.187-1.534	0.249			
Bilirubin	1.247	0.375-4.145	0.721			
Prothrombin time	0.984	0.951-1.019	0.371			
ICGR15	1.101	1.047-1.158	< 0.001	1.059	0.990-1.134	0.097
Tumor size	1.151	1.069-1.241	0.001	1.165	0.977-1.389	0.089
No. of tumors	1.075	0.728-1.588	0.723			
Absence of VI	0.232	0.109-0.496	< 0.001	0.228	0.092-0.568	0.002
Operative Time	1.003	0.999-1.006	0.189			
Blood loss	1.002	1.001-1.002	0.001	1.001	1.000-1.002	0.179
Blood transfusion	1.117	0.914-1.364	0.316			
Body mass index	0.957	0.839-1.091	0.509			
Platelets	1.023	1.000-1.047	0.109			
AST	1.024	1.012-1.037	0.001	1.010	0.994-1.026	0.209
ALT	1.005	0.992-1.019	0.472			
MELD	1.067	0.966-1.179	0.278			
BTR	0.772	0.557-1.068	0.084			
Hyaluronate	1.001	0.999-1.003	0.234			
HGF	43.179	2.321-803.29	0.015	52.366	1.310-2094.1	0.035
Alpha-fetoprotein	1.000	1.000-1.000	0.138			
PIVKA	1.000	1.000-1.000	0.534			

$P < 0.05$ was considered to be significant. N: Normal; CH: Chronic hepatitis; W: Well differentiated hepatocellular carcinoma; M: Moderately differentiated hepatocellular carcinoma; ICGR15: Indocyanine green retention rate at 15 min; VI: Vascular invasion; CI: Confidence interval; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; MELD: Model for end-stage liver disease; BTR: Branched chain amino acids to tyrosine ratio; HGF: Hepatocyte growth factor; PIVKA: Protein induced by vitamin K absence or antagonist.

as tumor size, tumor number, and vascular invasion^[27,28]. Our matched pairs exhibited similar liver function and tumor characteristics before hepatectomy. One possible reason why AR was associated with better OS than NAR in the matched pair analysis is that liver function was preserved better after AR than after NAR. The patients' tumor characteristics were matched by propensity score analysis, and the insignificant difference in RFS among the groups in the matched pair analysis demonstrates that this was successful. The AR procedure basically involves the resecting of the whole segmental area fed by portal blood flow; and hence, few or no necrotic non-functioning areas remain after the procedure^[13]. On the other hand, NAR only involves the resecting of the tumor margin and regions of the tumor that cross into other liver segments^[16]. Therefore, NAR might leave intact necrotic tissue or areas of hypo-perfusion in which liver function could deteriorate. If the resected liver volume had been similar in both groups, liver function might have been better after AR than after NAR due to the size of the ischemic area. However, we did not compare liver function or the total liver volume after liver resection between the AR and NAR groups. Most patients were discharged soon after hepatectomy without suffering any serious adverse events (data not shown). The above mentioned hypothesis should be examined in a future study.

AR eradicates putative intrahepatic occult metastases from HCC^[13,24]. Therefore, the local recurrence rate af-

ter AR might be lower than that after NAR. Indeed, the RFS curve of the AR was superior to that of the NAR within 24 mo after operation (RFS rate at 24 mo of AR was 59.7% *vs* that of NAR was 48.3%). However, RFS curves between the groups were becoming closer after 24 months after operation and the RFS periods of the AR and NAR groups eventually overlapped. Therefore, recurrence-free expectation of AR might be limited to within the early period after the operation. In addition, most recurrence was observed away from the resected segment in the NAR group (data not shown). This supports the hypothesis that HCC recurrence mainly involves multicentric tumor development rather than intrahepatic metastasis^[15-17]. Therefore, no apparent RFS difference was observed between the AR and NAR although the OS of the AR was significantly longer than that of the NAR.

Oncological behavior, such as the size and number of tumors, also plays an important role in the prognosis of HCC patients after initial hepatectomy^[29,30]. The Milan criteria^[31] represent the gold standard method for predicting prognosis not only after liver transplantation^[32] but also after liver resection^[33,34]. Most studies that found that AR was associated with favorable outcomes recruited patients with single tumors of less than 5 cm in diameter (who would meet the Milan criteria) who had maintained good liver function. Our results generally support the findings of these reports, but some patients in our study had more than one tumor. Hence, our results suggest that

the indications for AR for HCC should be extended from only patients with single tumors to include patients with two small tumors. Further study is needed to determine the exact number and size of tumors that predict a better clinical outcome after AR.

Although selection bias was inevitable in the matched pair analysis, we obtained an interesting result in our Cox proportional hazards model-based multivariate analysis; *i.e.*, we identified two independent prognostic factors among the matched pair cases. Vascular invasion had already been identified as a significant prognostic factor in HCC^[2,35,36]. The biological activity of HGF promotes the proliferation of both native^[37,38] and malignant cells^[39,40]. Both the serum HGF level and the incidence of HCC development increase with the progression of hepatitis and cirrhosis^[4,41,42]. This suggests that a relationship exists between tumor progression and HGF activity in HCC patients. Although this might be unique to the specific subgroup of patients examined in the present study, the blockade of this biological pathway might represent a target of molecular therapy for HCC.

We compared the post-hepatectomy prognosis of HCC patients between patients that underwent AR and those that underwent NAR. Propensity score analysis successfully matched subjects from each group with similar liver function levels and tumor characteristics. Although RFS did not differ significantly between the groups, the OS of the AR group was significantly longer than that of the NAR group. Therefore, AR for HCC conveys a survival advantage over NAR in patients with tumors of less than 5 cm in diameter, single tumor, and good liver function.

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COMMENTS

Background

The optimal type of liver resection for hepatocellular carcinoma (HCC) has been debated and is divided into anatomic resection (AR) and non-anatomic resection (NAR). Meta-analysis of AR vs NAR has demonstrated the superiority of AR in specific subgroups. However, none of these reports were randomized control studies. To overcome the effects of patient background, performing multivariate analysis followed by propensity score-matched analysis makes it possible to compare elective groups whilst minimizing confounding factors in non-randomized retrospective studies. The aim of this study is to elucidate the prognostic differences among AR and NAR for HCC after matching gender, tumor characteristics, and liver function using propensity score analysis.

Research frontiers

Propensity matched analysis could compare the groups who had similar background of the clinical factors and features. Although it allows us to compare specific subpopulations, it can be alternative for randomized control study. We could conclude our clinical interests in specific circumstances after propensity matched analysis if the number of recruiting patients were large enough to obtain statistical significance.

Innovations and breakthroughs

The authors compared the post-hepatectomy prognosis of HCC patients between patients that underwent AR and those that underwent NAR. Propensity score analysis successfully matched subjects from each group with similar liver

function levels and tumor characteristics. Although recurrence free survival did not differ significantly between the groups, the overall survival of the AR group was significantly longer than that of the NAR group.

Applications

AR for HCC conveys a survival advantage over NAR in patients with tumors of less than 5 cm in diameter, single tumor, and good liver function.

Terminology

AR is a resection of one or more segment which is characterized by Glisson's anatomy. On the other hand, NAR is a resection of the liver parenchyma regardless anatomic structure. AR tends to lose more liver parenchyma with liver proper function than NAR. If the liver function was maintained, AR was preferred to select for HCC resection. On the contrary, NAR was preferred if the liver function was deteriorated to avoid postoperative liver failure.

Peer review

This paper describes the prognosis comparison of HCC patients between patients that underwent AR and NAR using propensity score-matched populations. Further two independent prognostic factors have been found from multivariate analysis. This study provides the information for the prognostic advantage of AR in HCC patients.

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Detection of genotypic clarithromycin-resistant *Helicobacter pylori* by string tests

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(*H. pylori*) by polymerase chain reaction (PCR)-restriction fragment length polymorphism.

METHODS: Patients undergoing endoscopic examinations were enrolled in the present study. String tests were done on the next day of endoscopy. Segments of 23S rRNA were amplified from DNA obtained from string tests. PCR-restriction fragment length polymorphism was accomplished by restriction enzymes BbsI and BsaI recognizing the mutation site A to G at 2143 or at 2142 of 23S rRNA domain V, respectively.

RESULTS: One hundred and thirty-four patients with *H. pylori* infection underwent string tests. To compare phenotypic resistance, 43 isolates were successfully cultured in 79 patients in whom 23S rRNA was successfully amplified. Of five patients with clarithromycin-resistant *H. pylori*, 23S rRNA of *H. pylori* isolates from four patients could be digested by BsaI. In 38 susceptible isolates, 23S rRNA of *H. pylori* isolates from 36 patients could not be digested by either BsaI or BbsI. The sensitivity and specificity of the string test to detect genotypic clarithromycin resistance were 66.7% and 97.3%, respectively. Positive and negative predictive values were 80% and 94.7%, respectively.

CONCLUSION: String test with molecular analysis is a less invasive method to detect genotypic resistance before treatment. Further large-scale investigations are necessary to confirm our results.

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Key words: *Helicobacter pylori*; String test; Clarithromycin resistance; Polymerase chain reaction-restriction fragment length polymorphism

Core tip: According to region, antibiotic resistance is mostly detected by culture of endoscopic biopsy. The

Abstract

AIM: To evaluate the utility of the string test to detect genotypic clarithromycin-resistant *Helicobacter pylori*

study aimed to detect genotypic clarithromycin resistance by string tests. Amplified 23S rRNA from strings was digested by restriction enzymes to discriminate A2142G or A2143G mutations conferring clarithromycin resistance. Culture was also done to compare genotypic and phenotypic resistance. Sensitivity and specificity of the method were 66.7% and 97.3%, respectively. Positive and negative predictive values were 80% and 94.7%, respectively. Our study demonstrates that the string test, rather than endoscopic biopsy culture, could provide an option for molecular analysis in future.

Wu JY, Wang SSW, Lee YC, Yamaoka Y, Graham DY, Jan CM, Wang WM, Wu DC. Detection of genotypic clarithromycin-resistant *Helicobacter pylori* by string tests. *World J Gastroenterol* 2014; 20(12): 3343-3349 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i12/3343.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i12.3343>

INTRODUCTION

Since *Helicobacter pylori* (*H. pylori*) was isolated in 1984^[1], it has been widely believed to be a major cause of peptic ulcer, gastritis and mucosa-associated lymphoid tissue lymphoma (MALToma)^[1-4]. Evidence from Mongolian gerbils and epidemiological studies suggests the carcinogenesis of *H. pylori* in gastric cancer^[5,6]. It has been reported that increased production of reactive oxygen species and reactive nitrogen species by *H. pylori* leads to gastric inflammation and carcinogenesis^[7]. Recent reports showed that eradication of *H. pylori* not only reduced the severity and recurrence of peptic ulcers and gastritis, but also diminished the chance of gastric cancer development^[8-10]. Furthermore, remissions in MALToma were also proven after *H. pylori* was eradicated^[11,12]. Thus, eradication of *H. pylori* is the uppermost important issue in gastric ulcer, duodenal ulcer, MALToma, atrophic gastritis, and gastric adenocarcinoma, as well as following gastric cancer resection^[13].

Currently, several regimens for *H. pylori* eradication have been suggested, such as traditional triple, sequential, hybrid, and concomitant therapies^[14-18]. However, one major cause of unsuccessful eradication is the presence of antimicrobial resistance^[19]. In patients with metronidazole-resistant strains, 20% to 50% decreases in cure rates were noted with metronidazole-based combination regimens^[20,21]. In clarithromycin-based triple therapy, treatment failure has been reported in more than 50% of patients with clarithromycin-resistant strains^[22]. Therefore, early detection of antibiotic resistance could avoid treatment failure.

In regard to *H. pylori* antibiotic susceptibility tests (either agar dilution test or E-test), invasive endoscopic biopsy for culture of *H. pylori* isolates is necessary. However, technique-dependent culture procedures limit its clinical application for most general practices. Consequently, it is a practical issue to investigate more rapid and

less invasive methods to detect antimicrobial resistance prior to eradication therapy.

The mechanisms of antibiotic resistance in *H. pylori* have been widely studied in previous studies. For clarithromycin, point mutations at “hot-spots” (A to G at 2142, 2143) in 23S rRNA domain V were proposed as the major mechanism of clarithromycin resistance of *H. pylori*^[23]. With appropriate restriction enzymes (BbsI and BsaI), the mutations (A to G at 2142, 2143) were discriminated between susceptible and resistant strains by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)^[24].

It was reported that there were 10⁷-10⁸ organisms per milliliter of gastric juice^[25]. Therefore, *H. pylori* in gastric juice could be detected by non-invasive tests such as bacterial culture, rapid urease test (RUT), and polymerase chain reaction (PCR) assay^[26]. The string test was proven to be able to detect *H. pylori* with a high sensitivity and specificity^[27]. Approximately 0.5 mL of gastric juice with *H. pylori* attached by every 10 cm of the string was reasonable for molecular biological analysis^[28]. The aim of this study was to evaluate the string test to detect genotypic clarithromycin-resistant *H. pylori*.

MATERIALS AND METHODS

Patients undergoing endoscopic examinations at Kaohsiung Medical University Hospital were candidates in this study, which was approved by the local ethics committee. Patients who had taken antibiotics, bismuth salts, or proton pump inhibitors within one month, those who had ever received *H. pylori* eradication treatment or gastric surgery, and those who had a bleeding peptic ulcer, severe co-morbidity, or current pregnancy or lactation were all excluded. *H. pylori* infection was documented by the rapid urease test (RUT), culture, and histology in all enrolled patients. ¹³C-UBT was also performed on the same day. Positive *H. pylori* infection was considered when either culture yield was positive or any two of the other three tests, including RUT, histology, and ¹³C-UBT, were positive. The string test was carried out in patients with *H. pylori* infection on the following day.

The procedure of ¹³C-UBT was modified from a previous protocol^[29]. Briefly, the regimen consisted of ingestion of 100 mg ¹³C-urea agent (manufactured by the National Nuclear Institute of Taiwan) following 100 mL of milk as a test meal. An overnight fast for at least 8 h was requested. After ingestion of the ¹³C-urea agent, patients were asked to rinse their mouth out three times. Duplicate baseline breath samples were taken before ingestion, and 25 min after ingestion for the test. A mass spectrometry device was used to measure excess ¹³C in breath samples. The result of ¹³C-UBT more than 4/mL was defined as positive.

The string test (Entero-Test *H. pylori*, HDC Corporation, CA, United States) was used to detect *H. pylori* as previously described^[27]. A 90-cm nylon string coiled inside a gelatin capsule was used. A free-end looped string

Table 1 Polymerase chain reaction primers and sequences used in this study

Primer name	Sequence	Annealing temperature	Size (bp)
cagA	5'-GAT AAC AGG CAA GCT TTTGAC G-3'	50 °C	349
cagR	5'-CTG CAA AAG ATT GTT TGG CAG A-3'		
HP-K1	5'-CCA CAG CGA TGT GGT CTC AG-3'	54 °C	425
HP-K2	5'-CTC CAT AAG AGC CAA AGC CC-3'		

The annealing temperature and the size of amplicons are also listed. The reaction conditions are detailed in Materials and Methods.

protrudes through a hole in the other end of the capsule. Before the capsule was swallowed, 10-20 cm of the free-end string was pulled out and its position was ensured by adhesion of a small piece of tape to the patient's cheek. It was swallowed with 300 mL of water after 8 h of fasting. One hour after swallowing, the string was retrieved in a swift motion to prevent gag reflex and discomfort. The capsule separated from the string during withdrawal and was passed into the stool; although minimal complications such as capsule retention can occur, these did not happen in our study fortunately. The withdrawn string yielded one to two mL of gastric juice and was placed in a sterile petri dish without any fluid to prevent dilution. The first 30 cm was discarded to preclude oral contamination. The string was then checked against the pH indicator. The segment of string, showing low pH as red color appearance, was most desirable. The string was then processed for extraction of DNA, PCR, cultured for *H. pylori* and CLO test.

Genomic DNA was extracted from the string as previously described^[30]. To ensure the presence of *H. pylori* genes, the extracted DNA was first tested for the *cagA* gene by PCR, as the prevalence of the *cagA* gene of *H. pylori* in Taiwan is greater than 95%^[31]. The PCR condition and the sequence of the primers were used as previously indicated (Table 1)^[32]. Briefly, 20 µL of PCR mixture, containing 5 µL of extracted DNA, 200 µmol/L of (each) deoxynucleoside triphosphates (dNTPs), 0.4 µmol/L (each) primer, 1.5 mmol/L MgCl₂, and 1 U of Taq polymerase in PCR buffer [20 mmol/L Tris-HCl (pH 8.4), 50 mmol/L KCl, 0.2% glycerol], was held for 5 min at a denaturation temperature of 95 °C, followed by 35 cycles of 30 s each at a denaturation temperature of 95 °C, an annealing temperature of 50 °C, and an elongation temperature of 72 °C and by 5 min at 72 °C. The PCR products were analyzed by 1% agarose electrophoresis.

Among subjects with positive PCR amplification of the *cagA* gene, PCR-RFLP was done to elucidate the point mutations (A2142G and A2143G) of 23S rRNA, which were responsible for clarithromycin resistance of *H. pylori*^[24,33]. PCR primers and conditions used to amplify the fragments of the peptidyl transferase region of the 23S rRNA are listed in Table 1. In brief, PCR amplification of DNA was performed in a final volume of 50 µL containing 100 ng of *H. pylori* genomic DNA, 75 mmol/L Tris-HCl (pH 8.8), 20 mmol/L (NH₄)₂SO₄, 0.01%

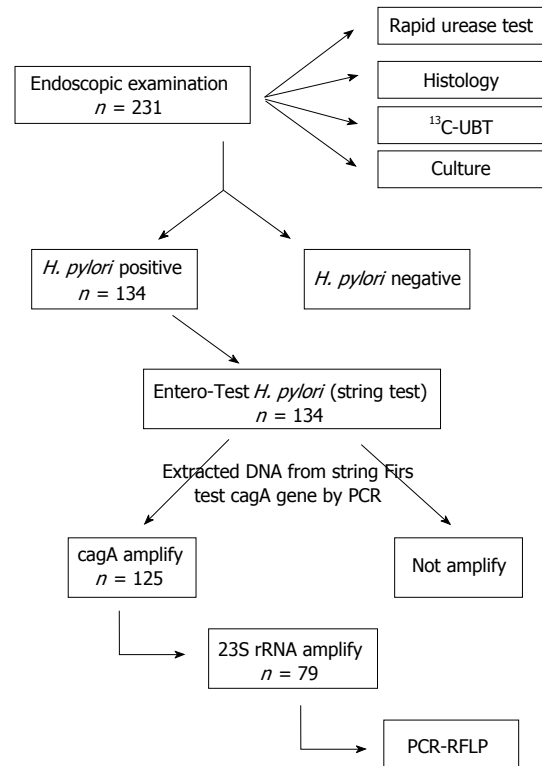


Figure 1 Trial profile. PCR: Polymerase chain reaction; RFLP: Restriction fragment length polymorphism; *H. pylori*: *Helicobacter pylori*.

Tween 20, 1.5 mmol/L MgCl₂, 0.2 mmol/L of dNTPs, 1 mol/L of primers and 2 U of Taq DNA polymerase. The cycling program was 1 cycle at 95 °C for 5 min; 35 cycles of 95 °C for 30 s, 54 °C for 30 s, and 72 °C for 30 s, and a final elongation step at 72 °C for 10 min. Ten microliters of amplicon was incubated with restriction enzymes (24 h at 56 °C for BsaI and at 37 °C for BbsI) to discriminate wild type, A2142G mutant (BbsI restriction site), and A2143G mutant (BsaI restriction site)^[24].

RESULTS

One hundred and thirty-four patients (58%) were proved to be infected with *H. pylori* by invasive and non-invasive methods as described in Materials and Methods. These patients underwent string tests on the next day of endoscopy. The trial profile is shown in Figure 1. To ensure that the retrieved string contained detectable amounts of *H. pylori*, PCR-based *cagA* gene detection was also done since the *cagA* gene is reported at a high detection rate in *H. pylori* from East Asia and Taiwan^[31,34], and *cagA* genes were detected from 93.3% of strings (125/134), validating the usability of DNA (Figure 2). Eventually, segments of 23S rRNA were amplified in 79 of 125 patients with positive gene amplifications from strings. To elucidate the point mutations (A2142G and A2143G) of 23S rRNA which are the main mechanism of clarithromycin resistance of *H. pylori*, restriction enzymes (BsaI and BbsI) were applied. Seventeen amplicons possess BsaI-recognizable restriction site (*i.e.*, A2143G mutant),

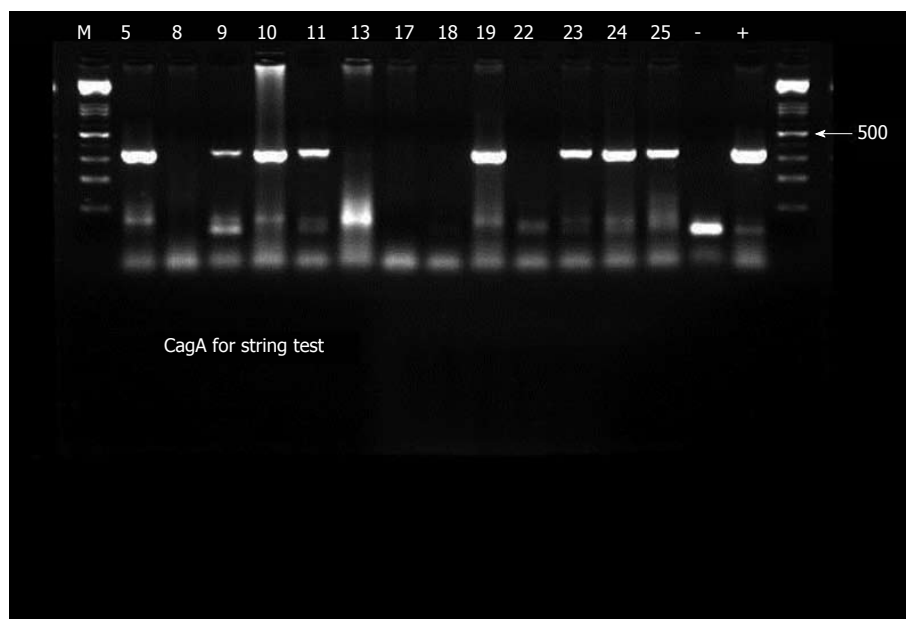


Figure 2 Polymerase chain reaction amplification of the *cagA* gene. The numbers on the top row indicate the patient numbers. M: Marker; -: Negative control; +: Positive control.

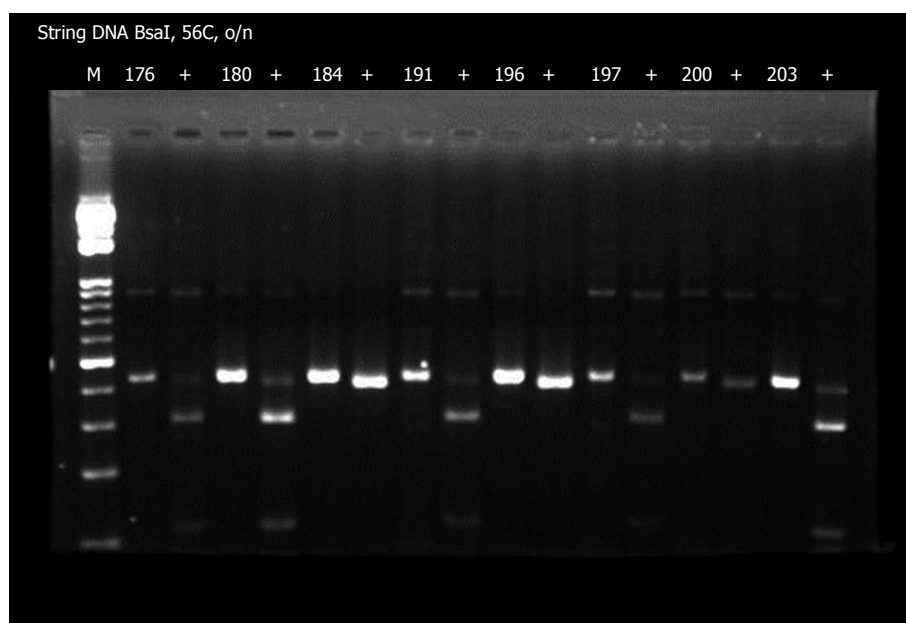


Figure 3 Polymerase chain reaction-restriction fragment length polymorphism for detection of clarithromycin resistance. The numbers on the top row indicate the patient numbers. M: Marker; +: Amplicons digested by BsaI. Clarithromycin-resistant *Helicobacter pylori* isolates from five patients (patient Nos. 176, 180, 191, 197, and 203) showed positive reactions.

whereas none of the 79 amplicons had the restriction site for BbsI (A2142G mutant) (Figure 3).

Culture from string is affected by several factors such as transportation, storage, and the contamination from oral flora, and is therefore more difficult than biopsied tissues. Among 79 patients who underwent string-based PCR-RFLP, *H. pylori* was successfully cultured in 43 patients. Among 43 *H. pylori* isolates, five (11.6%) were phenotypic-resistant to clarithromycin by E-tests and four were genotypic-resistant by BsaI digestion (*i.e.*, A2143G mutant). Otherwise, 36 isolates were not digested by

either BsaI or BbsI in 38 phenotypically susceptible *H. pylori* (Table 2). Therefore, the sensitivity and specificity of string-based PCR-RFLP to detect genotypic clarithromycin resistance of *H. pylori* were 66.7% and 97.3%, respectively. Positive and negative predictive values were 80% and 94.7%, respectively.

DISCUSSION

Since *H. pylori* eradication failure is caused mainly by antimicrobial resistance, detection of resistance prevalence

Table 2 Result of polymerase chain reaction-restriction fragment length polymorphism from the string test

		String PCR-RFLP	
		Sensitive	Resistant
E-test	Sensitive	36	2
	Resistant	1	4

Five were resistant to clarithromycin as confirmed by E-tests in 43 *Helicobacter pylori* isolates. Among these 43 patients undergoing the string test, four 23S rDNA amplicons from strings were digested by *BsaI*, indicating a mutation site A to G at 2142 of 23S rRNA. PCR-RFLP: Polymerase chain reaction-restriction fragment length polymorphism.

is critical for choice of treatment strategy^[22]. Among antibiotics for *H. pylori* eradication, clarithromycin resistance plays an important role in eradication failure when clarithromycin-based triple therapy is used^[19,35]. For susceptibility testing, *H. pylori* culture is always mandatory but not practical for most general practices due to technical difficulty. Therefore, empirical therapy was suggested by regional resistance prevalence^[13]. The current study aimed to develop a less invasive method for detection of genotypic clarithromycin resistance before “test-and-treat”^[36].

Clarithromycin resistance of *H. pylori* caused by single point mutations within 23S rRNA has been reported^[37]. A to G mutations at positions 2143 and 2144 of 23S rRNA were proposed as one of the major causes of clarithromycin resistance^[38]. Although other mutations (A2142C, A2143C, A2115G, G2141A, and A2142T) have also been reported to be associated with resistance to clarithromycin, studies from East Asian countries have shown that more than 90% of the mutant strains had the A2143G mutation instead of the A2142C mutation^[39]. Another study in China showed that gene mutation rates of A2142C, A2142G, and A2143G in the 23S rRNA gene were 1.5% (1/65), 6.2% (4/65), and 84.6% (55/65), respectively^[40]. Clarithromycin-resistant *H. pylori* with the A2143G mutation possesses a recognizable sequence (2143GAGACC2148) by restriction enzyme *BsaI*, whereas the sequence of resistant strains with the A2142G mutation is recognized by *BbsI*. Therefore, appropriate restriction enzymes (*BbsI* and *BsaI*) can be used to discriminate susceptible and resistant strains at “hot-spot” mutations (A2142G or A2143G)^[24].

Because bacterial culture is not always satisfactory, the string test was used to detect *H. pylori* as described in a previous report^[27]. This was a gastric juice-based PCR to detect the bacteria and tissue obtained by the string. According to the previous result, string test-based PCR for the detection of *H. pylori* was accurate, convenient, and well tolerated by patients. Besides the detection of *H. pylori*, it also carried approximately 0.5 mL of gastric juice containing PCR-detectable yields of bacteria absorbed by every 10 cm of the string. Therefore, the utility and efficiency of string tests for detection of *H. pylori* have been well established in several studies^[41,42].

High *cagA* detection rate (93.3%, 125 out of 134 patients) merited the usability of DNA from string for

providing further molecular analysis in the present study. In positive cases, 23S rRNA was successfully amplified in 79 cases whereas *H. pylori* was successfully cultured from strings in 43 patients. Among 43 isolates, five (11.6%) were resistant to clarithromycin with similar antibiotic resistance prevalence in the same region^[43]. 23S rRNA from string DNA of four patients was digested by *BsaI* among the five patients with clarithromycin-resistant *H. pylori*. DNA from 36 strings was not digested by either *BsaI* or *BbsI* in 38 patients with clarithromycin-susceptible *H. pylori*. In further analysis by bacterial DNA from culture, five resistant isolates possessed the A2143G mutation, which was compatible with previous reports that more than 90% of the resistant strains had the A2143G mutation in Asia^[44].

In conclusion, the sensitivity and specificity of string-based PCR-RFLP for detection of genotypic resistance of clarithromycin were 66.7% and 97.3%, respectively, in the present study. Positive and negative predictive values were 80% and 94.7%, respectively. Our study provided a possible option for less invasive genotypic analysis of clarithromycin resistance rather than culture of endoscopic biopsy. However, further large-scale investigations are necessary to confirm our results.

COMMENTS

Background

One major cause of unsuccessful *Helicobacter pylori* (*H. pylori*) eradication is the presence of clarithromycin resistance. Phenotypic resistance always requires susceptibility tests by culture. Evaluation of genotypic clarithromycin resistance is considered to have an important role for successful treatment. Therefore, the study was designed to validate the string test to detect genotypic clarithromycin-resistant *H. pylori*.

Research frontiers

By appropriate molecular analysis such as polymerase chain reaction-restriction fragment length polymorphism, the string test could be a clinically useful tool to detect genotypic clarithromycin-resistant *H. pylori*.

Innovations and breakthroughs

This paper is the first study to detect genotypic clarithromycin resistance by the string test. Several papers have been published to detect *H. pylori* by the string test, but none of them have discussed antimicrobial resistance. The innovations of our study provide an option for less invasive genotypic analysis such as antibiotic resistance surveillance.

Applications

By the methods in the study, genetic analysis of *H. pylori* can be achieved by the string test rather than technical-dependent culture of invasive endoscopic biopsy.

Terminology

Genotypic resistance: organisms possessing well-known genetic mutations leading to antimicrobial resistance are considered genotypically resistant. Phenotypic resistance: resistance of organisms to antibiotics as revealed by antibiotic susceptibility tests.

Peer review

This study presented a new method to examine clarithromycin-resistant *H. pylori*, which is most important for successful treatment. Its topic, aim and methods are very interesting and really attractive. Presentation and composition of the article are also sound. This study provides many possibilities for the string test in the genetic molecular analysis in future.

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Laparoscopic hepaticoplasty using gallbladder as a subcutaneous tunnel for hepatolithiasis

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Abstract

AIM: To investigate the feasibility, efficacy and safety of laparoscopic hepaticoplasty using gallbladder as subcutaneous tunnel and sphincter-of-Oddi preservation for hepatolithiasis.

METHODS: From January 2010 to July 2013, six patients with hepatolithiasis were treated at our institution. All the patients underwent laparoscopic surgery. The procedures included common hepatic duct exploration, stone clearance by fiberoptic choledochoscopy, hilar bile duct hepaticoplasty with preservation of the sphincter of Oddi, anastomosis between the hilar bile duct and neck of the gallbladder, and establishment of a subcutaneous tunnel with the gallbladder. Two patients underwent left lateral hepatectomy simultaneously. Clinical data including operation time, intraoperative blood loss, operative morbidity, hospital mortality, stone clearance, and recurrence rate were analyzed.

RESULTS: All patients successfully completed laparoscopic surgery. The mean length of hospital stay was 4.5 ± 0.9 d (range: 3-6 d). The mean blood loss of the hepatectomy was 450 mL (range: 200-700 mL), and the blood loss of the other four was 137 ± 151 mL

(range: 50-400 mL). The mean operative time was 318 ± 68 min (range: 236-450 min). The operative morbidity and hospital mortality were zero. The immediate stone clearance rate was 100%. All patients were followed up for an average of 17 mo (range: 7-36 mo). One of the six patients had abdominal mass with pain, and subcutaneous tunnel cholangiography showed severe gallbladder-biliary anastomotic stricture at 4 mo postoperatively. There was no stone recurrence and no cholangitis during follow-up.

CONCLUSION: Laparoscopic hepaticoplasty using gallbladder with a subcutaneous tunnel and preserving the sphincter of Oddi is feasible, safe and effective for hepatolithiasis.

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Key words: Hepatolithiasis; Laparoscopy; Hepaticoplasty; Minimally invasive surgery; Subcutaneous tunnel

Core tip: The treatment of hepatolithiasis is still a great challenge for surgeons. The residual and recurrent calculi are two major difficulties. This study introduces a new technique for hepatolithiasis and describes its two advantages. The first is that the sphincter of Oddi is preserved and it prevents intestinal reflux, which decreases the postoperative cholangitis rate; the second is that the subcutaneous tunnel is a minimally invasive approach for residual and recurrent stones that avoids reoperation. In selected cases, the operation can be completed *via* laparoscopy, and this technique is simple and safe.

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INTRODUCTION

Hepatolithiasis is prevalent in Southeast Asia but rare in Western countries. The occurrence rate was reported to be 0.6%-1.3% of patients with gallstones in Taiwan, South Korea and China^[1,2]. Long-term hepatolithiasis can lead to secondary biliary cirrhosis and cholangiocarcinoma, and may be a heavy burden on patients^[3-5]. In the past, the traditional treatment procedures for hepatolithiasis were common bile duct exploration, resection for fibrous and atrophic liver tissues, and choledochojunosotomy. In recent years, the development of laparoscopic surgery has introduced a minimally invasive method such as laparoscopic hepatectomy^[6]. Laparoscopic hepatectomy for the treatment of hepatolithiasis has been widely used in many institutes, but, to the best of our knowledge, there are no reports of laparoscopic hepaticoplasty with gallbladder for treatment of hepatolithiasis.

Since 1993, an open operative procedure has been introduced in which hepaticoplasty was performed using a free segment of jejunum or gallbladder for a subcutaneous tunnel and preservation of the sphincter of Oddi. The aim was to set up a tunnel between the biliary duct and tela subcutanea in order to remove recurrent stones and drain the bile in recurrent cholangitis in a minimally invasive manner^[7]. Since January 2010, a new laparoscopic surgical procedure has been attempted and the results are reported below.

MATERIALS AND METHODS

Clinical data

From January 2010 to July 2013, six patients (5 female and 1 male; mean age: 57.8 years, age range: 21-74 years) with hepatolithiasis underwent laparoscopic surgery at our institution. Ultrasonography (USG), computed tomography angiography and magnetic resonance cholangiopancreatography (MRCP) were performed preoperatively concerning the stricture location, distribution of calculi, concomitant liver fibrosis and atrophy, and the anatomy of the biliary tree and vascular system.

Indications for laparoscopy

The indications for laparoscopic surgery were: (1) fit for open surgery (good general condition, no significant organ dysfunction, able to tolerate anesthesia and liver resection); (2) no extrahepatic or future remnant intrahepatic biliary stricture, or no suppurative cholangitis; (3) Child-Pugh class A or B liver function without serious atrophy-hypertrophy complex or severe hepatic portal translocation; (4) no gallbladder size shrinkage and no pathological changes; and (5) no necessity for large hepaticoplasty because of small size of bile duct stricture. Diagnosis of cholangiocarcinoma by preoperative imaging, and intraoperative frozen section and postoperative

pathological examination findings were not taken into consideration.

Distribution of calculi

Three of the patients had stones in segments II-IV, one in segments V-VI, and two had bilateral stones. In one of the latter two patients, stones were accompanied by hilar bile duct stricture, and both had left lateral hepatic atrophy.

Laparoscopic bile duct exploration

Patients were placed in the Lloyd-Davis position under general anesthesia with tracheal intubation. The primary surgeon stood between the patient's legs, with one assistant on each side. A CO₂ artificial pneumoperitoneum was established with intra-abdominal pressure controlled at 12-14 mmHg. The sites of trocars were similar to those in the laparoscopic cholecystectomy. The length and site of the common hepatic choledochotomy used were governed by the size and location of the stones, which was at least 3 cm. Detection of intra- and extra-hepatic bile ducts was carried out by flexible fiberoptic choledochoscopy (4.9 mm P-20; Olympus, Tokyo, Japan) through the choledochotomy to find and remove the stones and to detect any bile duct strictures. Stones were extracted by forceps, stone basket or saline flushing under choledochoscopic guidance. Large impacted stones were fragmented by plasma shock wave lithotripsy^[8] and extracted by choledochoscopy.

Laparoscopic left hepatectomy

A new trocar was placed at the right subcostal midclavicular line. The round, falciform, left coronary and triangular ligaments were transected with a harmonic scalpel (Olympus). The margin of hepatectomy ran for 1 cm to the left side of the falciform ligament and hepatic transection was continued with the harmonic scalpel to the inferior side of the liver. The hepatic parenchyma was transected gradually along the transection line with the harmonic scalpel or Ligasure (Covidien, Mansfield, MA, United States). The pedicles of hepatic segments II and III were exposed and transected with titanium clips, hemlock or EndoCutter (Covidien). The left hepatic vein was transected with a linear EndoCutter, and the specimen was placed into the specimen bag. A flexible fiberoptic choledochoscope was used to detect extra- and intrahepatic bile ducts by the orifice of the left hepatic bile duct, remove stones in the hepatic bile duct (Figure 1), and close the orifice by suture. Finally, the raw surface was examined carefully to ensure that there was no bleeding or bile leakage.

Laparoscopic hepaticoplasty using gallbladder as a subcutaneous tunnel

The gallbladder was examined carefully to ensure that its wall was normal. The cystic duct was exposed, cut off and ligated, the cystic artery was preserved to avoid injury, and the neck of the gallbladder was freely separated.

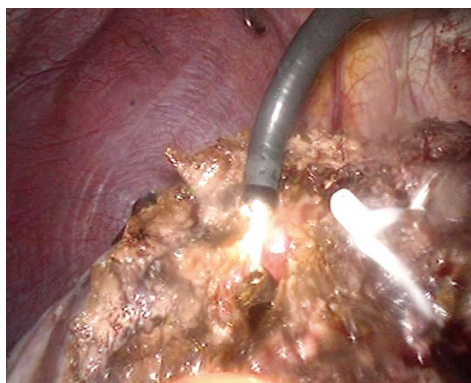


Figure 1 Removal of intrahepatic duct stones by stone basket using a flexible fiberoptic choledochoscope.

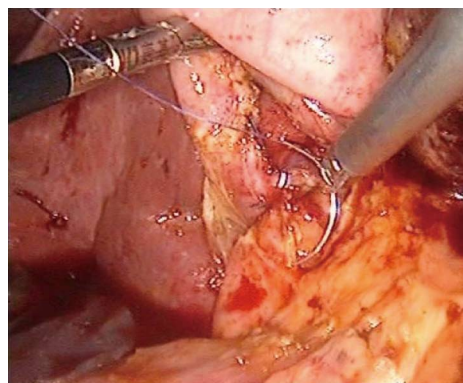


Figure 3 Hepatic duct was repaired using the neck of the gallbladder.

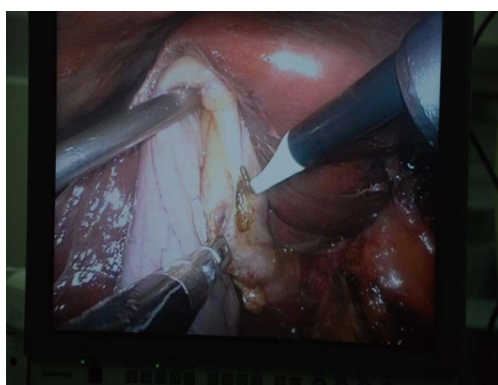


Figure 2 Neck of the gallbladder was opened longitudinally about 3 cm in length.



Figure 4 Fundus of gallbladder was fixed at the tela subcutanea to establish a tunnel between the hepatic duct and the subcutaneous layer.

The neck of the gallbladder was opened longitudinally at about 3 cm in length (Figure 2); a flexible fiberoptic choledochoscope was inserted into the gallbladder to detect whether the mucosa was normal, and to remove gallbladder stones. If the mucosa was normal, the gallbladder was used as a tunnel. For hilar bile duct stricture, the common hepatic duct was opened longitudinally, stricture of the right and left hepatic ducts was corrected, and the hepatic duct was repaired using the neck of the gallbladder (Figure 3). Side-to-side anastomosis was performed. The gallbladder bed was separated to ensure that the fundus of the gallbladder could be drawn and fixed at the peritoneum and abdominal rectal muscle anterior sheath (Figure 4). The fundus of the gallbladder was about 3 cm × 2 cm on rectus sheath. Finally, the gallbladder fundus was marked on the skin with electrocautery.

For all cases, a T-tube was routinely inserted into the common bile duct for postoperative cholangiography and choledochoscopy.

Follow-up

All patients received the same postoperative care by the same team of surgeons. USG was conducted every 3-6 mo, and then annually, or whenever the patients presented with symptoms suggestive of cholangitis. MRCP or endoscopic retrograde cholangiopancreatography was

performed if USG showed features of stone recurrence or ductal strictures. If necessary, cholangiography was performed by opening the subcutaneous channel.

RESULTS

All patients successfully completed laparoscopic surgery. The mean length of hospital stay was 4.5 ± 0.9 d (range: 3-6 d). The mean blood loss of the hepatectomy was 450 mL (range: 200-700 mL), and the blood loss of the other four was 137 ± 151 mL (range: 50-400 mL). The operative time was 318 ± 68 min (range: 236-450 min). The operative morbidity and hospital mortality were zero. The immediate stone clearance rate was 100%.

All patients were followed up for an average of 17 mo (range: 7-36 mo). There was no stone recurrence or cholangitis during follow-up. One patient had an abdominal mass with pain, and subcutaneous tunnel cholangiography showed severe gallbladder-biliary anastomotic stricture at 4 mo postoperatively. Considering the subcutaneous tunnel failure, we performed gallbladder chemical ablation with 95% ethanol^[9,10] via the subcutaneous tunnel because the patient refused cholecystectomy.

DISCUSSION

Hepatolithiasis is still endemic in East and Southeast

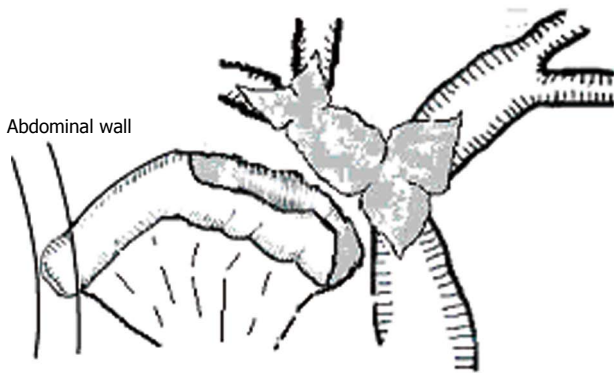


Figure 5 Anterior wall of the hepatic basin was repaired using a free segment of jejunum, and a hepatico-subcutaneous stoma was formed.

Asia, and is characterized by bile duct stricture and stone formation, causing acute and recurrent life-threatening cholangitis. Repeated cholangitis results in bacterial infection in the biliary system, liver and blood, and leads to liver abscess, sepsis, biliary cirrhosis and even cholangiocarcinoma. To prevent immediate and late sequelae, aggressive treatment is needed.

There are many methods of treatment for hepatolithiasis, such as bile duct exploration and hepatectomy. All of them have the same principles: to remove lesions, extract stones, correct strictures, maintain drainage, and prevent recurrence. In recent years, minimally invasive treatment of hepatolithiasis has developed greatly, including laparoscopy, choledochoscopy, and percutaneous transhepatic choledochoscopy.

Many authors have reported that hepatectomy seems to be the most definitive approach for hepatolithiasis to remove the lesions including stones, strictures, dilation and affected hepatic tissues^[11-13]. Laparoscopic hepatectomy has also become possible with the availability of new instruments that allow bloodless liver resection. In 1996, Azagra *et al.*^[14] were the first to report laparoscopic hepatic left lateral lobectomy. Since then, there have been many cases of laparoscopic hepatectomy for hepatolithiasis reported, confirming the good short- and long-term effects, high safety, rapid recovery, even for patients with previous biliary operations^[15-19].

Although some cases of regional-type hepatolithiasis can benefit from hepatectomy, residual and recurrent stones are still a major challenge for diffuse-type hepatolithiasis. The reasons may include: (1) the lesions of diffuse-type hepatolithiasis are only reduced through hepatectomy and another treatment approach is needed; (2) it is difficult to remove stones thoroughly during surgery for diffuse-type hepatolithiasis, and the stone recurrence rate is high^[12]; and (3) postoperative reflux cholangitis related to the method of biliary reconstruction, especially Roux-en-Y hepaticojejunostomy, has become an important cause of stone recurrence^[20,21].

Reoperation for residual and recurrent hepatolithiasis is difficult, and can impose great physical and mental suffering on patients. After several years of experimental

and clinical research, we established a new technique for treatment of residual and recurrent hepatolithiasis and have achieved good results. We performed hepaticoplasty with gallbladder or segmental free jejunum for a subcutaneous tunnel and preserved the sphincter of Oddi. There are two advantages to this technique for residual and recurrent hepatolithiasis: preservation of the sphincter of Oddi and the use of a subcutaneous tunnel.

In recent years, surgeons have paid increasing attention to the preservation of the sphincter of Oddi. The sphincter consists of the ampulla, biliary sphincter and pancreatic sphincter. It regulates the excretion of bile and pancreatic juice, prevents intestinal reflux, and avoids retrograde biliary infection. If the function of the sphincter of Oddi is destroyed, there is a series of pathological changes, such as decreased basal pressure of the sphincter, disappearance of the common bile duct-duodenum pressure gradient, biliary pneumatosis, bacteria in the bile juice, and bile duct chronic inflammation. Thus, many complications such as recurrent bile duct stones, cholangitis and cholangiocarcinoma occur^[22]. Some authors have reported that patients with sphincter of Oddi dysfunction tend to have a higher risk of recurrence and a greater demand for reoperation than those without these conditions^[23-25]. Therefore, we suggest that the sphincter of Oddi should be preserved if there is no laxity or restriction.

The key point of this technique is to ensure the normal function of the sphincter of Oddi. There are many methods to diagnosis sphincter of Oddi dysfunction, but many of them are invasive^[26]. We have found a relatively simple method for intraoperative judgment: if the intraoperative exploration shows that a 16 Fr catheter can pass through the orifice of the duodenum ampulla, the sphincter of Oddi can be considered to have normal function and it should be preserved.

The second feature of this technique is the use of a subcutaneous tunnel as a minimally invasive approach for treatment of residual or recurrent hepatolithiasis. The first case of hepaticoplasty using the jejunum with a subcutaneous tunnel was performed at our institute in 1993. The common hepatic and left and right hepatic ducts were opened longitudinally to form a “basin of hepatic duct”, then a 12-15-cm free pedicled jejunum (anal side) was side-to-side anastomosed with the “basin of hepatic duct” and the oral side of the jejunum was fixed to the abdominal wall (Figure 5).

The gallbladder subcutaneous tunnel is more physiological and convenient than the free jejunum. If no gallbladder shrinkage or pathological changes are found, and there is no need for large-scale hepaticoplasty because of the small size of bile duct stricture, the gallbladder can be used as a subcutaneous tunnel. The anastomosis is not difficult even by laparoscopy. During follow-up, the tunnel can be incised for biliary drainage, cholangiography, choledochoscopic exploration, lesion biopsy, stones clearance, and biliary stricture dilation under local anesthesia whenever the patients present with symptoms suggestive

of cholangitis. Also, the tunnel can be marked by electrocautery intraoperatively or by USG postoperatively.

According to our experience, 24% (32/146) of recurrent or residual hepatolithiasis cases underwent biliary drainage, stone clearance and stricture dilation *via* the subcutaneous tunnel^[4]. The high availability enabled the patients to avoid reoperation and fully demonstrated the necessity and value of the subcutaneous tunnel.

In our follow-up, we did not find infection, rupture, volvulus, or increasing stone recurrence rate due to the subcutaneous tunnel. Although the gallbladder-hilar bile duct anastomosis stricture in the present study may have been due to inexperience, we have not found efferent obstruction of the subcutaneous tunnel in any of the other cases.

The difficulty of this technique is the total laparoscopic anastomosis of the gallbladder neck and hilar bile duct. It is necessary to dissect the triangle of Calot carefully, freeing the gallbladder bed, ligating the cystic duct and creating an anastomosis. It also requires intermittent all-layer suture at least 3 cm in diameter.

In summary, this technique reduces postoperative reflux cholangitis rate by preserving the sphincter of Oddi but also provides a minimally invasive treatment for recurrent or residual stones *via* the subcutaneous tunnel. Also, this technique is simple and safe. However, currently the number of cases is small, and more cases and the long-term effects need further study.

COMMENTS

Background

Hepatolithiasis is prevalent in Southeast Asia, especially in China, South Korea and Taiwan, and aggressive treatment is needed because of the sequelae such as biliary cirrhosis and cholangiocarcinoma. The major difficulties of traditional therapies are serious trauma and high incidence of residual and recurrent stones.

Research frontiers

Many studies have shown that hepatolithiasis is a segmental disease along the biliary tree and hepatectomy seems to be the most effective method of treatment. Although laparoscopic hepatectomy is widely used and has achieved good results in localized hepatolithiasis, therapy for diffuse-type hepatolithiasis and the high incidence of residual and recurrent stones are still major challenges. The high incidence of reflux cholangitis caused by traditional hepaticojejunostomy is the major sequela during follow-up. In this study, the authors tried to find a new approach for effective and minimally invasive therapy for hepatolithiasis.

Innovations and breakthroughs

The authors established a minimally invasive approach for hepatolithiasis. The hepatic lesions could be resected laparoscopically, with simultaneous construction of a subcutaneous tunnel. The subcutaneous tunnel enabled them to remove residual or recurrent stones and avoid reoperation. The incidence of intestinal reflux cholangitis caused by traditional hepaticojejunostomy could be avoided by preserving the sphincter of Oddi.

Applications

By introducing the technique of laparoscopic hepatectomy and creating a subcutaneous tunnel, this study may present a strategy for treatment of diffuse-type hepatolithiasis and patients with a high risk of residual or recurrent stones.

Terminology

Hepatolithiasis is located above the confluence of the left and right hepatic ducts, and is a segmental disease that is strictly distributed along the biliary tree. It can lead to secondary biliary cirrhosis and cholangiocarcinoma. The high incidence of residual or recurrent stones and reflux cholangitis are major challenges.

Peer review

This is a well-written original paper describing an interesting and intelligent technique (hepaticoplasty using the gallbladder) for laparoscopic management of the rare and difficult entity of hepatolithiasis. Satisfactory results are reported.

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Retrospective analysis of adjuvant chemotherapy for curatively resected gastric cancer

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Abstract

AIM: To determine the efficacy of adjuvant chemotherapy for gastric cancer in clinical practice, a retrospective analysis was conducted in a high-volume Chinese cancer center.

METHODS: Between November 1995 and June 2007, a total of 423 gastric or esophagogastric adenocarcinoma patients who did (Arm A, $n = 300$) or did not (Arm S, $n = 123$) receive radical gastrectomy followed by postoperative chemotherapy were enrolled in this retrospective analysis. In Arm A, monotherapy (fluoropyrimidines, $n = 25$), doublet (platinum/fluoropyrimidines, $n = 164$), or triplet regimens [docetaxel/cisplatin/5FU (DCF), or modified DCF, epirubicin/cisplatin/5FU (ECF) or modified ECF, etoposide/cisplatin/FU, $n = 111$] were administered. Disease-free survival (DFS) and overall

survival (OS) were compared between the two arms. A subgroup analysis was carried out in Arm A. A multivariate analysis of prognostic factors was conducted.

RESULTS: Stage I, II and III cancers accounted for 9.7%, 35.7% and 54.6% of the cases, respectively, according to the American Joint Committee on Cancer (AJCC) staging system, 7th edition. Only 178 (42.1%) patients had more than 15 lymph nodes harvested. Hazard ratio estimates for Arm A compared with Arm S were 0.47 ($P < 0.001$) for OS and 0.59 ($P < 0.001$) for DFS. The 5-year OS rate was 52% in Arm A vs 36% in Arm S ($P = 0.01$); the adverse events in Arm A were mild and easily controlled. Ultimately, 73 patients (26.5%) who received doublet or triplet regimens switched to monotherapy with fluoropyrimidines. The OS and DFS did not differ between monotherapy and the combination regimens, however, both were statistically improved in the subgroup of patients who were switched to monotherapy with fluoropyrimidines after doublet or triplet regimens as well as patients who received ≥ 8 cycles of chemotherapy.

CONCLUSION: In clinical practice, platinum/fluoropyrimidines with adequate treatment duration is recommended for stage II/III gastric cancer patients according to the 7th edition of the AJCC staging system after curative gastrectomy even with limited lymphadenectomy.

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Key words: Adjuvant chemotherapy; Gastric cancer; Lymphadenectomy; Fluoropyrimidine; Platinum

Core tip: Although the ACTS GC and CLASSIC trials demonstrated that postoperative chemotherapy improved overall survival after standard D2 gastrectomy, severe challenges in adjuvant settings remain unsettled, such as low D2 resection rates in some regions. Our retrospective study is complementary to large-scale phase III prospective trials, and demonstrated the efficacy and

safety of postoperative platinum/fluoropyrimidines in stage II/III gastric cancer patients according to the updated 7th edition staging system after curative gastrectomy with standard or limited lymphadenectomy.

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INTRODUCTION

Gastric cancer (GC) is the fourth most common type of cancer and the second leading cause of cancer-related death worldwide^[1]. It is also the second most frequent malignancy in China^[2]. More patients are diagnosed with late stage GC in China than in South Korea and Japan, with up to 60% of patients in stage III according to the 7th edition of the American Joint Committee on Cancer (AJCC) staging system^[3,4].

Surgical resection of the primary tumor and regional lymph node dissection is the mainstay of curative treatment for patients with locally advanced GC (LAGC). Different types of surgical procedures for GC can affect the results of postoperative chemotherapy. Gastrectomy with extended (D2) lymphnode dissection is considered standard treatment in both Asian and Western countries^[5]. However, in clinical practice, D2 lymphadenectomy rates vary among different hospitals and regions in China. In some European and American countries such as Turkey and Chile, high incidence rates, high rates of late-stage GC, and deficiencies in specially trained surgeons are considered to be severely challenging as in China^[1].

Globally, adjuvant treatment varies among countries, based on data from different clinical studies. The Intergroup-0116 study and the MAGIC study showed that postoperative chemoradiotherapy or perioperative chemotherapy improved overall survival compared with surgery alone^[6,7]. However, both studies assessed the benefits of adjuvant therapy after only limited surgery, which has long been questioned by Asian oncologists. Recently, two large-scale randomized phase III trials (the ACTS GC study and the CLASSIC study) demonstrated that postoperative chemotherapy increased the 5-year overall survival (OS) rate by 13%-15% after standard D2 gastrectomy^[8,9]. However, significant challenges in adjuvant therapy remained unsettled, such as low D2-resection rates in many regions. Moreover, it is unclear whether the Japanese regimen with TS1 for 1 year or the Korean regimen with oxaliplatin/capecitabine (XELOX) for 8 cycles was more effective and better tolerated. To date, no direct comparison has been carried out in prospective studies, and oncologists are uncertain about which regimen to choose. In addition, the AJCC staging system was updated from the 6th to the 7th edition in 2010, but

neither of the above-mentioned trials enrolled sufficient patients with pathological grade T4 or N3 tumors. These patients were classified as stage IV under the 6th version, but were classified as stage II-III C under the 7th version^[10,11]. Thus, no evidence is available to guide adjuvant treatment in this population. It is clear that the data from the prospective ACTS GC and CLASSIC studies do not fully meet the needs in clinical practice, even in Japan and South Korea.

Thus, in this 12-year retrospective study, we assessed the benefit of adjuvant chemotherapy in LAGC patients classified according to the 7th edition of the AJCC system after curative gastrectomy with limited or standard lymphadenectomy.

MATERIALS AND METHODS

Between November 1995 and June 2007, 423 consecutive LAGC patients treated with surgery alone or with surgery followed by post-operative chemotherapy were enrolled in this study. The surgeries had been conducted by surgical oncologists or general surgeons in 62 different Chinese institutes ranging from specialized cancer centers to general hospitals, while the consultations or adjuvant chemotherapy had been carried out in a single center at the Department of Gastrointestinal Oncology, Peking University Cancer Hospital and Institute. The inclusion criteria were as follows: histologically confirmed gastric adenocarcinoma; curative resection with at least D1 lymphadenectomy; no evidence of distant metastases; TNM stage of I B-III C (according to the 7th edition of the AJCC staging system); no previous malignancies; and no neoadjuvant chemotherapy or radiotherapy prior to surgery. The exclusion criteria were as follows: incomplete medical records or refusal to follow-up.

Of the 423 enrolled patients, 123 received surgery alone (surgery-alone arm, Arm S) and 300 received post-operative adjuvant chemotherapy (adjuvant arm, Arm A). The chemotherapeutic regimens were as follows: monotherapy with fluoropyrimidines (capecitabine, TS-1, tegafur-uracil, infusional 5FU), doublet regimens (cisplatin with a fluoropyrimidine, oxaliplatin with a fluoropyrimidine), or triplet regimens (either paclitaxel, docetaxel, epirubicin or etoposide with cisplatin or oxaliplatin and a fluoropyrimidine). No patient received radiotherapy. Regimen selection was based on stage, performance status, available combinations and patient preferences. Triplet regimens were considered for patients with T3/T4 tumors and positive lymph nodes; doublet regimens were considered for patients with T3/T4 tumors or positive lymph nodes. Etoposide was administered prior to 2003, and taxanes were administered after 2003. Monotherapy was considered for patients with poor performance status or co-morbidities or elderly patients.

Patients in Arm A underwent hematologic testing and assessment of their clinical symptoms each week at Peking University Cancer Hospital and Institute. Patients in Arm S underwent examinations at local hospitals, and the results of their hematologic tests or symptom

records were not complete. Adverse events were defined according to the Common Toxicity Criteria of the National Cancer Institute, version 3.0. The presence of a relapse was determined by means of imaging studies or pathological diagnosis, including ultrasonography, computed tomography (CT), gastrointestinal radiography series, or endoscopy. For suspected disease, additional diagnostic tools were considered. Patients underwent at least one type of imaging study, usually CT, at 3-mo intervals during the first 2 years after surgery and at 6-mo intervals thereafter until 5 years after surgery.

Statistical analysis

The data were processed using SPSS version 15.0 for Windows XP. Disease-free survival (DFS) was defined as the time from surgery prior to a recurrence of gastric cancer, the occurrence of a second primary cancer, or death from any cause. OS was defined as the time from surgery to death from any cause. Univariate analyses were applied to evaluate the prognostic factors affecting the survival rate in patients with various histopathologic characteristics and adjuvant therapy regimens. Each categorical variable was compared using the chi-squared test. The Kaplan-Meier method was used for survival analysis. The Log-rank rule was applied in the monovariate analyses, while a Cox proportional hazard regression model was used in the multivariate analysis. A *P* value of less than 0.5 was considered statistically significant.

RESULTS

Patient characteristics

A total of 423 patients were enrolled in this study: 300 in Arm A and 123 in Arm S. In these patients, stage I, II and III GC accounted for 9.7%, 35.7% and 54.6%, respectively. As the surgeries had been carried out at various Chinese hospitals and no photographs were collected, the details of the procedures were difficult to qualify, however, 178 (42.1%) patients had more than 15 lymph nodes harvested.

The patient profile and tumor characteristics, except for age, were well balanced between Arm A and Arm S (Table 1). More elderly patients (≥ 65 years old) were included in Arm S (43.9%) than in Arm A (29.3%, *P* = 0.04). In addition, Arm S patients tended to have earlier-stage GC than Arm A patients (18.7% stage IB in Arm S *vs* 6.0% in Arm A, *P* = 0.12), and the rate of having a ratio of positive lymph nodes harvested < 0.33 was 66.7% in Arm S and 57.7% in Arm A (*P* = 0.07).

Adverse events, treatment compliance and modifications

Only the data for the 300 patients in Arm A were analyzed for adverse events, and the 123 patients in Arm S were not included in the safety analysis. Adverse events, including hematologic and non-hematologic toxic effects, were analyzed, and included leukopenia, anemia, thrombocytopenia, elevated total serum bilirubin levels, peripheral neuropathy, nausea, and vomiting. The most

frequent grade 3 or 4 adverse events were neutropenia (17.6%), nausea and vomiting (6.1%), anorexia (3.5%), and diarrhea (2.3%). In general, 61 patients (20.3%) developed grade 3 or 4 toxicities (data not shown).

Among the 300 patients in Arm A, the number of chemotherapy cycles ranged from 1 to 17 with a median of 6. Treatment was continued for at least 3 cycles in 269 patients (90.0%), at least 6 cycles in 176 patients (58.7%), at least 8 cycles in 79 patients (26.3%), and at least 10 cycles in 39 patients (13.0%). Reasons for withdrawal of treatment included refusal by the patient to continue treatment due to adverse events or other factors, the detection of metastasis or relapse. A total of 141 patients (47.0%) had dose modifications or chemotherapy delays. Of the 275 patients receiving doublet or triplet regimens, 73 patients (26.5%) switched to monotherapy due to toxicity or upon their request.

OS and DFS

By the last follow-up examination on May 1st 2010, 283 patients (66.9%) were confirmed to have recurrent disease, and 238 patients (56.3%) had died; only 6 patients (1.4%) were lost of follow-up. The median OS and DFS based on a median follow-up time of 87.0 mo were 56.2 (95%CI: 48.4-64.0) and 33.4 (95%CI: 25.3-41.5) mo, respectively. The 5-year survival rate was 48.0%. Both median OS and DFS were statistically longer in Arm A than in Arm S: the OS was 63.0 mo (95%CI: 46.7-79.3) *vs* 42.9 mo (95%CI: 37.4-48.3) (*P* = 0.001), respectively; the 5-year OS was 52% *vs* 36%, respectively (*P* = 0.01); and the DFS was 41.5 mo (95%CI: 24.4-58.6) *vs* 24.4 mo (95%CI: 15.7-33.1), respectively (*P* = 0.007) (Figure 1). For stage II/III patients, a similar survival benefit was observed in Arm A. In Arm A *vs* Arm S, the OS was 58.0 mo (95%CI: 48.4-67.6) *vs* 37.6 mo (95%CI: 30.3-44.9), respectively (*P* < 0.001); the 5-year OS was 52% *vs* 36%, respectively (*P* = 0.01); the DFS was 34.9 mo (95%CI: 22.2-47.6) *vs* 14.9 mo (95%CI: 16.0-22.0), respectively (*P* < 0.001). The 5-year DFS was 45% in the chemotherapy group and 28% in the surgery-alone group (*P* = 0.07).

Subgroup analysis of OS and DFS

Among the 45 patients over 65 years old, no benefit in OS was observed in Arm A (*n* = 32) compared with Arm S (*n* = 13). The DFS tended toward improvement with chemotherapy at 49.4 mo in Arm A (95%CI: 35.7-63.1) *vs* 29.8 mo in Arm S (95%CI: 24.0-35.6, *P* = 0.053). In each of the following subgroup analyses, an initial comparison was performed for patients over 65 to exclude potential bias due to age.

Patients in Arm A received monotherapy (*n* = 25) or doublet (*n* = 164) or triplet (*n* = 111) regimens as postoperative chemotherapy. The OS was shorter in the monotherapy group (46.6 mo, 95%CI: 25.6-67.6) than in the doublet (63.2 mo, 95%CI: 22.9-103.5) or triplet (65.2 mo, 95%CI: 43.4-86.9) therapy groups, but the difference was not statistically significant. The DFS showed the

Table 1 Characteristics of the patients *n* (%)

Characteristics	Total	Arm A	Arm S	<i>P</i> value
Number	423	300 (70.9)	123 (29.1)	NA
Sex				
Male	320 (75.7)	223 (74.3)	97 (78.9)	0.324
Female	103 (24.3)	77 (25.7)	26 (21.1)	
Age group				
< 65	281 (66.4)	212 (70.7)	69 (56.1)	0.04
≥ 65	142 (33.6)	88 (29.3)	54 (43.9)	
Histology (adenocarcinoma)				
Well-moderate differentiated	97 (22.9)	61 (20.3)	36 (29.2)	0.145
Poorly differentiated	287 (67.8)	211 (70.3)	76 (61.8)	
Signet-ring cell	24 (5.7)	18 (6.1)	6 (4.9)	
Mucinous	15 (3.5)	10 (3.3)	5 (4.1)	
Location of tumor				
Proximal	147 (34.8)	95 (31.7)	52 (42.3)	0.07
Distal	276 (65.2)	205 (68.3)	71 (57.7)	
Extent of LN dissection				
< 15	202 (47.8)	136 (45.3)	66 (53.7)	0.12
≥ 15	221 (52.2)	164 (54.7)	57 (46.3)	
Depth of invasion (T stage)				
T1	9 (2.1)	7 (2.3)	2 (1.6)	0.529
T2	67 (15.8)	35 (11.7)	32 (26.0)	
T3	204 (48.2)	165 (55.0)	39 (31.7)	
T4a	89 (21.0)	52 (17.3)	37 (30.1)	
T4b	54 (12.8)	41 (13.7)	13 (10.6)	
No. of invaded LN (N stage)				
N0 (0)	117 (27.7)	70 (23.3)	47 (38.2)	0.11
N1 (1-2)	126 (29.8)	100 (33.3)	26 (21.1)	
N2 (3-6)	95 (22.5)	68 (22.7)	27 (22.0)	
N3 (≥ 7)	85 (20.1)	62 (20.7)	23 (18.7)	
AJCC stage (7.0 version)				
I B	41 (9.7)	18 (6.0)	23 (18.7)	0.12
II A	100 (23.6)	70 (23.3)	30 (24.4)	
II B	51 (12.1)	38 (12.7)	13 (10.6)	
III A	56 (13.2)	48 (16.0)	8 (6.5)	
III B	98 (23.2)	67 (22.3)	31 (25.2)	
III C	77 (18.2)	59 (19.7)	18 (14.6)	
Positive/harvested LN ratio				
< 0.33	253 (59.8)	171 (57.0)	82 (66.7)	0.07
≥ 0.33	170 (40.2)	129 (43.0)	41 (33.3)	
Vascular invasion				
Positive	167 (39.5)	115 (38.3)	52 (42.3)	0.542
Negative	130 (30.7)	91 (30.3)	39 (31.7)	
Unknown	126 (29.8)	94 (31.4)	32 (26.0)	

LN: Lymph node; NA: Not available; AJCC: American Joint Committee on Cancer.

same tendency for the monotherapy, doublet and triplet therapy groups at 24.5 mo (95%CI: 8.2-40.8), 38.4 mo (95%CI: 20.0-80.3) and 45.8 mo (95%CI: 19.2-72.4), respectively ($P = 0.321$).

In the doublet regimen group ($n = 164$), 124 patients (75.6%) received oxaliplatin/fluoropyrimidines, and 40 patients (24.4%) received cisplatin/fluoropyrimidines; no differences in OS or DFS were detected between the two subgroups (data not shown). In the triplet regimen group ($n = 111$), 24 patients (21.6%) received DCF or modified DCF (taxanes/cisplatin/5FU), 52 patients (46.8%) received epirubicin/cisplatin/5FU (ECF) or modified ECF (epirubicin with cisplatin or oxaliplatin and 5FU or capecitabine), and 35 patients (31.5%) received etoposide/cisplatin/5FU. No differences in DFS or OS were observed among the three subgroups (data not shown). In Arm A, a total of 272 (90.7%) patients received platinum/

fluoropyrimidine-containing regimens which included either oxaliplatin ($n = 126$) or cisplatin ($n = 146$), and no survival differences were observed (data not shown).

Among patients who received doublet or triplet regimens, 73 patients (26.5%) switched to monotherapy with fluoropyrimidines, either oral or infused. Significant differences in the total number of chemotherapy cycles, OS and DFS were observed between patients who switched and patients who did not (Table 2, Figure 2). Regimens modified to monotherapy with fluoropyrimidines significantly prolonged OS and DFS in both the doublet and triplet regimen groups.

Given that switching to monotherapy could have improved the treatment tolerability and prolonged duration of the chemotherapy, we further compared survival data of patients who received ≥ 8 chemotherapy cycles within 8 mo after surgery with patients who received ≤

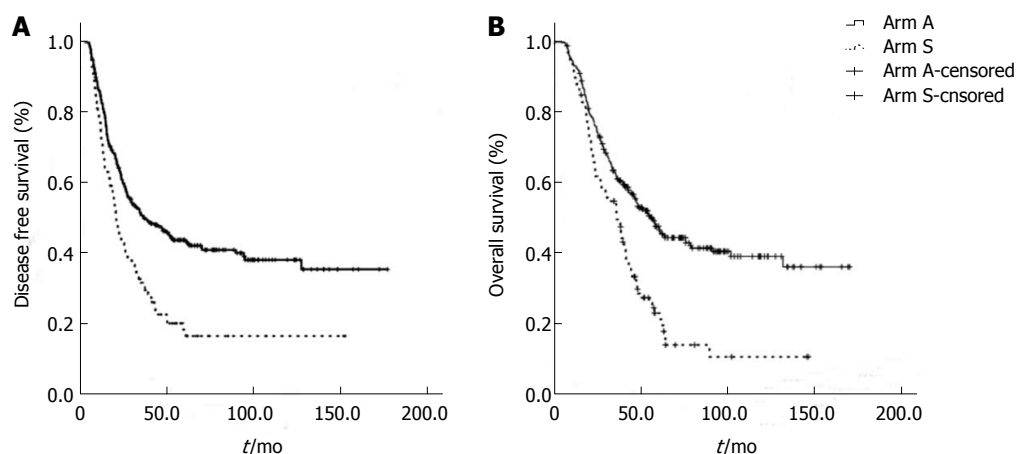


Figure 1 Kaplan-Meier curves of disease-free survival (A) and overall survival (B) in Arm A and Arm S. *P*-value by Log-rank test. A: Disease-free survival (DFS): 41.5 mo vs 24.4 mo, *P* = 0.007; B: Overall survival: 63.0 mo vs 42.9 mo, *P* = 0.001.

Table 2 Disease-free survival and overall survival of patients in Arm A received different chemotherapy regimens (*n* = 300)

	<i>n</i>	Cycles median (range)	Disease-free survival (mo) (95%CI)	Overall survival (mo) (95%CI)
Monotherapy	25	4 (2-15)		
Doublet	164	6 (1-17)	38.4 (21.0-80.3)	63.2 (22.9-103.5) ¹
Monotherapy switched	38	8 (3-17)	NR ¹	NR ¹
No monotherapy switched	126	6 (1-12)	25.4 (18.7-32.1)	44.4 (28.3-60.5)
Triplet	111	6 (2-14)	45.8 (19.2-72.4) ¹	65.2 (43.5-87.0) ¹
Monotherapy switched	35	9 (5-14)	NR ¹	NR ¹
No monotherapy switched	76	6 (2-11)	24.9 (9.4-40.4)	56.2 (42.0-70.4)
Doublet and triplet	275	6 (1-17)	45.8 (23.8-67.8)	63.8 (41.4-86.2)
Monotherapy switched	73	8 (3-17)	NR ¹	NR ¹
No monotherapy switched	202	6 (1-12)	25.4 (18.3-32.5)	49.4 (35.7-63.1)

¹Statistically significance (*P* < 0.001). NR: Not reached.

7 chemotherapy cycles in the same time period (Figure 3). Statistically longer OS and DFS rates were observed in the group with ≥ 8 chemotherapy cycles (*P* < 0.001), indicating that a longer adjuvant duration provided a survival benefit in patients who switched to monotherapy.

Univariate and multivariate analysis of prognostic factors

Univariate analysis showed an association between OS and DFS and location of the tumor (*P* = 0.014), T stage (*P* < 0.001), N stage (*P* < 0.001), positive/harvested lymph node (LN) ratio (*P* < 0.001), and adjuvant chemotherapy treatment (*P* = 0.001). Similarly, LN dissection was a significant factor for DFS (*P* = 0.032) (data not shown). In contrast, gender, age, WHO performance status and histological differentiation did not affect the OS or DFS.

On multivariate analysis, the extension of the LN dissection (< 15 and ≥ 15 LNs harvested), N stage and adjuvant chemotherapy were associated with OS and DFS, whereas the location of the tumor and T stage were independent factors for DFS. Therefore, the multivariate analysis using a Cox regression identified 3 prognostic factors: the extension of the LN dissection (*P* = 0.008), the N stage (*P* = 0.012), and treatment with postoperative chemotherapy (*P* < 0.001). After adjust-

ment, the Cox hazard ratio (HR) estimation for Arm A compared with Arm S was 0.47 (95%CI: 0.36-0.63; *P* < 0.001) for the OS and 0.59 (95%CI: 0.44-0.79; *P* < 0.001) for the DFS (data not shown), indicating a risk reduction in patients who received adjuvant therapy.

DISCUSSION

Adjuvant chemotherapy after curative resection is known to improve outcomes in gastric cancer treatment, although the preferred recommendations differ by geographical region^[12]. Based on the United States Intergroup-0116, United Kingdom MAGIC, Japan ACTS GC, and South Korea CLASSIC studies, the recommended adjuvant treatments are chemoradiotherapy in the United States, perioperative chemotherapy in the United Kingdom and a few other European countries, and adjuvant chemotherapy in most Asian countries, either TS1 for 1 year or XELOX for 8 cycles over 6 mo^[6-9]. The former two studies enrolled patients who underwent only limited surgeries, while the latter two studies enrolled patients who underwent at least D2 gastrectomy.

It is well accepted that the type of surgical procedure will affect the results of adjuvant treatment^[13]. D2 gastrectomy is now recommended as the standard surgical

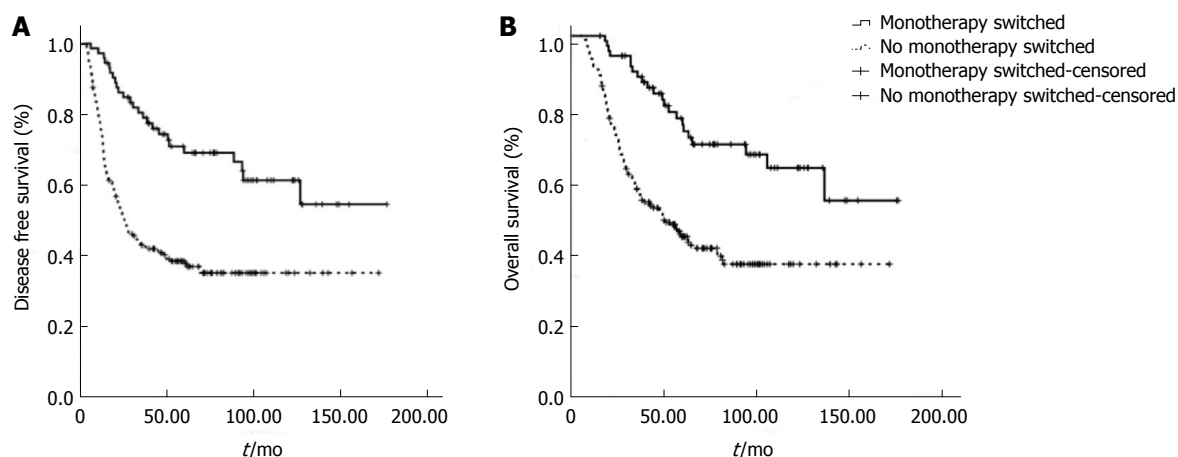


Figure 2 Kaplan-Meier curves of disease-free survival (A) and overall survival (B) in patients with monotherapy switched or not. *P*-value by Log-rank test. A: Disease-free survival: Not reached vs 25.4 mo, *P* = 0.000; B: Overall survival: Not reached vs 49.4 mo, *P* = 0.005.

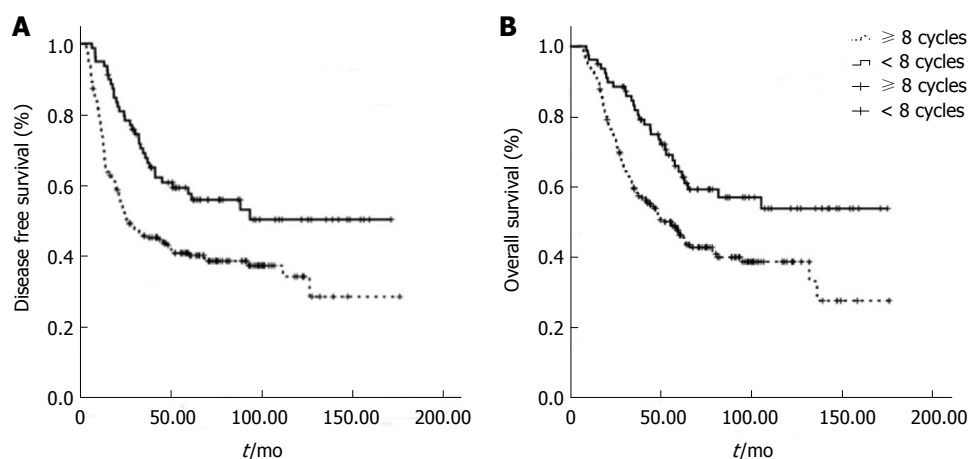


Figure 3 Kaplan-Meier curves of disease-free survival (A) and overall survival (B) in patients treated with ≥ 8 cycles or not. *P*-value by Log-rank test. A: Disease-free survival: Not reached vs 25.8 mo, *P* = 0.001; B: Overall survival: Not reached vs 56.1 mo, *P* = 0.002.

treatment for resectable GC in both Asian and Western countries^[14-17]. However, D2 lymphadenectomy is a demanding technique, requiring rigorous training and a sufficient number of annual operations to ensure the skill of the surgeons. Unlike Japan and South Korea, many institutions in rural areas or small cities in China are not specialized centers with appropriate surgical expertise and postoperative care. In 2010, out of 2312 consecutive GC patients who underwent resection in a high-volume cancer center, more than 14 lymph nodes were harvested in only 650 (28.1%)^[18]. Additionally, although D2 lymphadenectomy has become more widespread recently in China due to continuing education, the exact proportion of D2 lymphadenectomies throughout the country is not yet available. In this study, it was difficult to establish the details of the surgical procedures at other institutions. We were only able to qualify the surgeries by the number of lymph nodes harvested based on the classification of the NCCN guidelines in which dissecting a minimum of 15 lymph nodes for histologic examination is required for both D1 and modified D2 resections^[15].

In addition, T4/N3 patients classified according to the

6th edition of the AJCC staging system are now classified as stage II-III C in the updated 7th edition. Although they lacked distant metastasis, these patients were previously regarded as stage IV and thus were not enrolled in clinical studies of adjuvant therapies such as the ACTS GC and CLASSIC studies. However, these so-called “stage IV-M0” patients comprise a significant fraction of the patients in China. Therefore, these two large-scale phase III studies are still far from solving the known challenges in clinical practice such as non-ideal surgeries and more patients at later stages. We hope that this retrospective study which conducted analyses using the definitions of the 7th edition of the AJCC staging system will provide complementary data for oncologists not only in China, but also in nations where D2 lymphadenectomy is limited to some extent. Patients were consecutively enrolled at a single center, but they were drawn from throughout China for consultation or treatment. Thus, the findings should be applicable to clinical practice nationwide.

Age was not well balanced between the two arms of surgery alone and surgery with adjuvant chemotherapy, with more elderly patients in Arm S. However, in the sub-

group of patients over 65 years of age ($n = 45$), no differences in the OS or DFS were observed between Arm S and Arm A. It is unlikely that the age imbalance influenced the results of the overall analysis. Patients in Arm S tended to be at earlier pathological stages, although this trend was not statistically significant. In general clinical practice, oncologists are less likely to prescribe chemotherapy for older patients or patients at relatively earlier stages because requests from patients and their families would interfere with the doctors' decisions under such circumstances.

In general, adjuvant chemotherapy in this 12-year retrospective study was safe and effective in prolonging the 5-year OS and DFS. Following the exclusion of stage I patients, the survival benefit remained significant in stage II/III patients according to the 7th edition of the AJCC staging system. This benefit was also confirmed on multivariate analysis, which showed a risk reduction in patients who received adjuvant therapy. Currently, no further data on adjuvant chemotherapy in prospective studies using the 7th edition of the AJCC staging system are available. Based on this study, it is reasonable to deduce that patients classified as stage II/III under the new staging system are also likely to benefit from postoperative chemotherapy.

Surprisingly, no significant difference in survival was found among monotherapy and doublet and triplet regimens, while patients who switched to monotherapy or underwent ≥ 8 cycles of chemotherapy experienced prolonged DFS and OS by up to 2-fold. These results were consistent with the findings from the ACTS GC study in which the OS correlated with the duration of TS1 administration^[19]. Considering that only 67% of the patients completed adjuvant chemotherapy in both the ACTS GC and CLASSIC studies, proper timing of treatment modification and an adequate duration of adjuvant treatment might be more effective in producing survival benefit than high-dose chemotherapy or combinations of stronger or more chemotherapeutic agents.

Subsequent to fluoropyrimidines and platinum, paclitaxel sequenced with oral fluoropyrimidines was tested in Yoshida's large-scale randomized phase III study (the SAMIT trial), but it failed to show a survival benefit superior to monotherapy with oral fluoropyrimidines^[20]. In our study, various chemotherapeutic agents, including taxane-, platinum-, epirubicin-, or etoposide-based regimens, did not show any significant differences in survival benefit. These results were supported by an inter-trial comparison between the ARTIST and CLASSIC studies^[21]. After standard D2 gastrectomy, patients receiving 6 cycles of cisplatin/capecitabine, the control group in the ARTIST study, showed a 3-year DFS, which was similar to patients receiving 8 cycles of oxaliplatin/capecitabine in the CLASSIC study. Consequently, fluoropyrimidines with or without platinum (either cisplatin or oxaliplatin) is considered effective and safe as adjuvant therapy, even for patients who did not receive standard D2 lymphadenectomy. New agents such as taxanes and even trastuzumab for HER2-overexpressing tumors should be studied in future explorative trials based on

molecular pathological classification systems or the efficacy of predictive biomarkers.

In conclusion, this retrospective study was complementary to large-scale phase III prospective trials which demonstrated the efficacy and safety of postoperative platinum with fluoropyrimidines in stage II/III gastric cancer patients under the updated 7th edition AJCC staging system after curative gastrectomy with standard or limited lymphadenectomy. Necessary treatment modifications and adequate treatment durations are recommended in adjuvant settings.

COMMENTS

Background

Adjuvant chemotherapy after curative resection is known to improve outcomes in gastric cancer treatment, although the preferred recommendations differ by geographical region.

Research frontiers

Recently, two large-scale randomized phase III trials demonstrated that postoperative chemotherapy increased the 5-year overall survival rate by 13%-15% after standard D2 gastrectomy. However, significant challenges in adjuvant therapy remained unsettled, such as low D2-resection rates in many regions.

Innovations and breakthroughs

In clinical practice, platinum/fluoropyrimidines with adequate treatment duration is recommended for stage II/III gastric cancer patients under the 7th edition of the American Joint Committee on Cancer staging system after curative gastrectomy with even limited lymphadenectomy.

Applications

Adjuvant chemotherapy in this 12-year retrospective study was safe and effective in prolonging the 5-year overall survival and disease-free survival.

Peer review

It's an interesting and well-presented retrospective study demonstrating the efficacy and safety of postoperative platinum/fluoropyrimidines.

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Multi-slice computed tomography manifestations of hepatic epithelioid angiomyolipoma

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Abstract

AIM: To explore the characteristics of multi-slice computed tomography (CT) manifestations of hepatic epithelioid angiomyolipoma (HEA), improve the rate of accurate diagnosis, and reduce the misdiagnostic rate.

METHODS: The multi-slice CT manifestations in five patients who were diagnosed with HEA definitely by postoperative pathological examination were analysed retrospectively. Three female patients and two male patients were included. Before operation, four patients received plain CT scanning and dynamic enhancement scanning, and the other patient only received enhancement scanning, with immunohistochemical analysis conducted after postoperative pathological examination. Four patients were misdiagnosed by CT, including three patients misdiagnosed with hepatic cell carcinoma and one patient with focal nodular hyperplasia.

RESULTS: Upper abdominal multi-slice spiral CT scanning and three-stage enhancement scanning were conducted in five patients with HEA before operation. HEA had certain characteristic CT manifestations: low density masses, a few relatively high-density masses or fat-density masses diffusely shown in foci, clear boundary, round or oval and large focus, and tumour size ranging from 3.1 cm × 2.5 cm to 7.0 cm × 5.2 cm. During enhancement scanning, the foci were significantly enhanced uniformly or non-uniformly during the arterial phase, while during the venous and equilibrium phases, the foci were enhanced continuously or showed obvious low-density masses. Obviously enhanced and widened vessels could be found adjacent to foci or in the central area of foci during the arterial phase.

CONCLUSION: CT manifestations of HEA have certain characteristics. Primary diagnosis can be obtained by combining CT findings with clinical data, but pathological examination is still needed for a definite diagnosis.

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Key words: Liver; Epithelioid; Angiomyolipoma; Computed tomography

Core tip: The multi-slice computed tomography manifestations in five patients with HEA were analyzed in the study. In computed tomography scanning, low density masses, a few relatively high density masses or fat density masses were showed in foci, the boundary was clear, the focus was round or oval and large, and the size of the tumour was 3.1 cm × 2.5 cm to 7.0 cm × 5.2 cm. The foci were significantly enhanced during arterial phase, enhanced continuously or showed obvious low density masses during venous phase and equilibrium phase, and obviously enhanced and widened vessels could be found adjacent to foci or in the central area of foci.

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INTRODUCTION

Angiomyolipoma commonly occurs in the kidney and liver, but is also found in the retroperitoneum, heart, mediastinum, lung, and vagina^[1-3]. A typical angiomyolipoma consists of vessels, smooth muscles, and fat. Ishak^[4] reported hepatic epithelioid angiomyolipoma (HEA) for the first time. HEA is a kind of rare mesenchymal benign tumour and a special type of angiomyolipoma. HEA consists of epithelioid cells and is commonly found in females, with the male to female ratio of 1:5^[5,6]. HEA does not have special clinical manifestations and may be easily confused with many kinds of tumours. Therefore, the rate of misdiagnosis is very high.

At present, HEA is considered a kind of epithelioid tumour adjacent to vessels and different from hamartoma^[7,8]. The pathological diagnosis depends on immunohistochemical examination^[9]. The biological behaviour of HEA is benign generally, but malignant HEA has been reported, and the prognosis of some patients are bad. Now many scholars have realised that the biological behaviour of HEA has malignant potential^[10-14]. Some studies have shown that high expression of Ki-67 is one of the characteristics of HEA^[15]. Therefore, early diagnosis of HEA is very important.

Multi-slice spiral computed tomography (MSCT) is an important method for preoperative diagnosis of HEA, but the CT manifestations are various because HEA does not contain or rarely contains mature adipose tissue^[16-18]. Thus, diagnosis is difficult, with the accuracy rate of preoperative diagnosis being 32% or less, and diagnosis mainly depends on puncture and biopsy currently^[19,20]. This study retrospectively analysed CT manifestations in five patients who were diagnosed with HEA definitely by pathological examination and immunohistochemical examination, in order to improve diagnosis of the disease and provide a reference for improving the rate of accurate preoperative diagnosis of HEA.

MATERIALS AND METHODS

Subjects

Five patients with HEA who were diagnosed definitely by pathological examination after operation at our hospital from May 2010 to June 2012 were included, including two men and three women. Their mean age was 56.5 years (range: 46-61 years). Three patients were found with a liver space-occupying lesion and without any clinical symptoms; two patients visited the hospital because of distending pain in the right upper quadrant without obvious causative factors. All five patients were found to be

HBsAg- and alpha fetoprotein-negative and have normal hepatic function. Before operation, four patients received plain CT scanning and dynamic enhancement scanning, and the other patient only received enhancement scanning.

Methods

All patients were asked to comply with an absolute diet for 6-8 h before scanning. The patients were supine when scanning was performed, using 64-MSCT (Siemens, Germany). The scanning parameters were as follows: the spiral collimation was 64×0.625 ; the thickness of each slice was 5 mm; the interval thickness was 5 mm; the speed of couch movement was 12 mm/s; the tube voltage was 120 kV; and the tube current was 260-320 mAs. The extent of scanning was as follows: during the plain scanning phase, arterial phase, venous phase, and equilibrium phase, the extent of scanning was from the diaphragmatic dome to the level of the inferior pole of the kidney, including the whole liver; the scanning was finished for one time when the patient held their breath at the end of inspiration. Anconal venous transfusion of non-ionic contrast medium (OmniPaque 300 or Ultravist 300, 80-100 mL) with a high pressure injector was used during enhancement scanning. The rate of injection was 3.0 mL/s. The starting times of the arterial phase, venous phase, and equilibrium phase were 25, 35, and 60 s after the beginning of injection, respectively. After scanning, the original data were processed by thin slice reconstruction (one millimetre, the interval between two adjacent slices was one millimetre). The images of all patients received multiplanar reconstruction.

RESULTS

Upper abdominal multi-slice spiral CT scanning and three-stage enhancement scanning were conducted in the five patients before operation. The growth positions of HEA included the right lobe of the liver (three patients) and the left lobe of the liver (two patients). The size of tumour was 3.1 cm \times 2.5 cm to 7.0 cm \times 5.2 cm. The CT manifestations of HEA were: the tumours were round; slightly low-density masses were found in four patients (Figure 1A) and a low-density mass was found in one patient (Figure 2A); the boundary of tumours was clear; and no bleeding, necrosis, cystic degeneration, or calcification was found. A little fatty density was found in one case, but dilatation of adjacent intrahepatic bile duct was not found.

The manifestations during dynamic enhancement scanning were: the lesions of five patients were enhanced significantly during the arterial phase; and the enhancement degree was stronger than that of liver parenchyma, including four patients with non-uniform enhancement (Figure 1B) and one patient with uniform enhancement (Figure 2B). Contrast agent disappearance was found in three patients during the venous phase and equilibrium phase, including two patients with an obvious decrease of

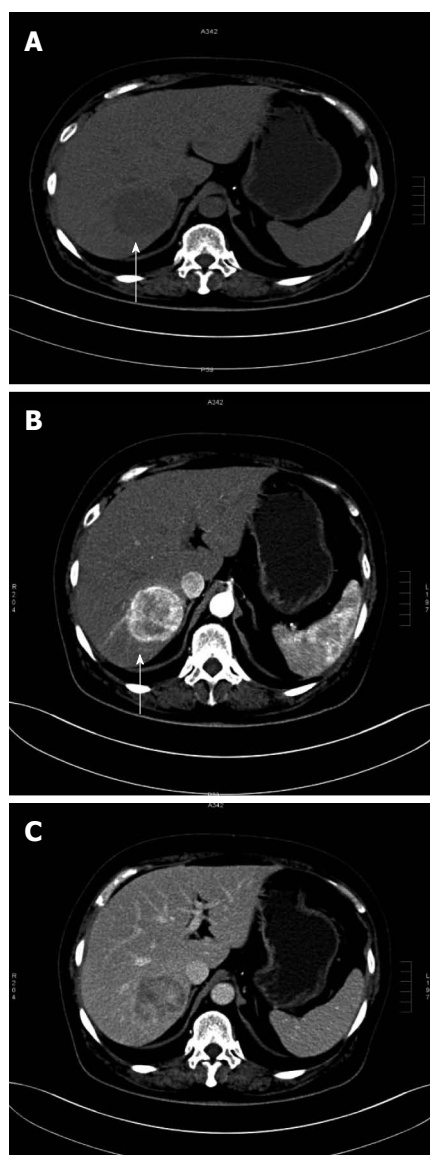


Figure 1 Computed tomography plain scanning: oval mass in posterior segment of the right hepatic lobe, slightly low density, clear boundary, and uniform density (A); venous phase: the enhancement of foci weakening (B); arterial phase: obvious and uniform enhancement of foci (C).

enhancement degree, which was significantly lower than that of normal liver parenchyma, and one patient with a decrease of enhancement degree that was slightly lower than that of normal liver parenchyma (Figure 1C). The lesions of two patients were enhanced continuously. The enhancement degree of lesions during the venous phase was stronger than that of normal liver parenchyma, and the enhancement degree of lesions during the equilibrium phase was similar to that of liver parenchyma (Figure 2C). Enlarged vessels were found near the tumour or in the central area of the tumour of two patients (Figure 1B).

Among the five patients with HEA, four were misdiagnosed, including three diagnosed with hepatic cell carcinoma (HCC) and one with focal nodular hyperplasia (FNH). Multi-slice CT manifestations in five patients with HEA are displayed in Table 1.



Figure 2 Arterial phase: non-uniform enhancement of foci (indicated by a white arrow), and widened vessels in central area of foci (indicated by a black arrow) (A); CT plain scanning: oval mass in the right hepatic lobe, low density, clear boundary, and uniform density (B); equilibrium phase: continuous enhancement of foci, and equidensity (C).

DISCUSSION

HEA is a kind of very rare mesenchymal tumour and mainly consists of epithelioid cells^[3]. HEA does not contain adipose tissue or contains little mature adipose tissue, so its diagnosis is difficult. In recent years, it has been reported that angiomyolipoma is a true neoplasm^[20], but there are previous few reports on imaging of HEA, and the prognosis of some patients is bad^[21,22]. Therefore, more attention should be paid to the disease. In this study, the diagnosis of HEA was improved by retrospectively analysing the imaging data of five patients with HEA.

CT manifestations of HEA vary and diagnosis is difficult. Most of the patients were diagnosed definitely by puncture and biopsy^[23,24]. Peh and partners^[25] reported

Table 1 Multi-slice computed tomography manifestations in five patients with hepatic epithelioid angiomyolipoma

Shape	Boundary	Density	Enhancement	Abnormal vessels adjacent to tumors and intratumoral abnormal vessels
Oval	Clear	Slightly low, uniform	Fast in and slow out enhancement	No
Oval	Clear	Slightly low, non-uniform, containing a few lipid	Fast in and fast out enhancement pattern, non-uniform enhancement	No
Oval	Clear	Slightly low, uniform	Fast in and fast out enhancement pattern, non-uniform enhancement	Yes
Oval	Clear	Slightly low, uniform	Fast in and slow out enhancement pattern, continuous and non-uniform enhancement	No
Oval	Clear	Slightly low, uniform	Fast in and slow out enhancement pattern, continuous and non-uniform enhancement	Yes

three patients with HEA of different manifestations, including one case whose CT manifestations were similar to those of FNH, one case whose CT manifestations were similar to those of HCC, and one case with typical CT manifestations of HEA (coexistence of lipid, widened vessels, and smooth muscle tissue, which were similar to the reported manifestations). It is easy to diagnose typical HEA mixed with lipid, but most of the HEA foci had no lipid, which made diagnosis difficult^[5].

Analysis of CT manifestations in five patients with HEA showed that the boundary of foci of the five patients was clear, the foci were large, round or oval. The densities in four patients were relatively uniform and without lipid; the density in one patient was not uniform, with a little adipose tissue in focus. During enhancement scanning, the foci of five patients were obviously enhanced during the arterial phase, including the foci of four patients that were enhanced non-uniformly and the focus of one patient that was enhanced uniformly. The vessels adjacent to foci or in the central area of foci widened. During the venous phase, four patients showed low-density masses, and one patient showed a high-density mass. This mass continued to be present in the equilibrium phase, showing equidensity mass.

It is concluded that it is not difficult to diagnose the lesions with adipose density in foci, while for the lesions without adipose density in foci, HEA should be considered when the following conditions appear: low density is shown in plain CT scanning, the boundary is clear, the foci are round or oval, and the size of foci is large (the long diameter of foci in the study was 3.1 cm or more); during enhancement scanning, the foci are obviously enhanced uniformly or non-uniformly during the arterial phase, then the foci show high density or obvious low density during the venous phase and equilibrium phase; and obviously enhanced and widened vessels are found in the area adjacent to foci or in the central area of foci during the arterial phase.

Among the five patients with HEA in the study, three showed low-density masses or slightly low-density masses during plain scanning, and they were misdiagnosed as HCC because of the characteristic of ‘fast in and fast out enhancement pattern’; and one patient showed a slightly low-density mass during plain scanning, and the patient was misdiagnosed with FNH because of the characteris-

tic of ‘fast in and slow out enhancement pattern’. Thus, HEA needs to be differentiated from the following diseases.

HCC

Patients with HCC often show an irregular low-density mass, and most of them have medical histories of hepatitis B or liver cirrhosis. The level of alpha foetoprotein is high, and portal vein cancer embolus formation is often found. Large foci are enhanced obviously and non-uniformly during enhancement scanning. In contrast, HEA is a round or oval mass with slightly low or low density. The foci are large with a ‘fast in and fast out enhancement pattern’, and the enhancement of foci is not uniform generally. The patients do not have a medical history of hepatitis B and the results of laboratory examination are negative^[23-25].

FNH

The foci are equidensity and equisignal masses compared with liver parenchyma on CT. The characteristic is a star scar in the central area of foci, which show a ‘fast in and slow out enhancement pattern’ and continuous enhancement, but the star scar cannot be enhanced. Conversely, the foci of HEA are slightly low-density or low-density masses, without a scar in the central area of foci^[23-25].

In short, the imaging manifestations of HEA have certain characteristics. Primary diagnosis can be obtained by combining CT findings and clinical data (*e.g.*, without a medical history of hepatitis B and negative results of laboratory examination), but a definite diagnosis depends on puncture and biopsy or histopathological examination.

COMMENTS

Background

Hepatic epithelioid angiomyolipoma (HEA) is a kind of very rare mesenchymal tumour and mainly consists of epithelioid cells. HEA does not contain adipose tissue or contains little mature adipose tissue, so its diagnosis is difficult. In recent years, it has been reported that angiomyolipoma is a true neoplasm, but there are previous few reports on imaging of HEA, and the prognosis of some patients is bad. Therefore, more attention should be paid to the disease. In this study, the diagnosis of HEA was improved by retrospectively analysing the imaging data of five patients with HEA.

Research frontiers

In recent years, it has been reported that angiomyolipoma is a true neoplasm,

but there are previous few reports on imaging of HEA, and the prognosis of some patients is bad. Therefore, more attention should be paid to the disease.

Innovations and breakthroughs

In this study, the diagnosis of HEA was improved by retrospectively analysing the imaging data of five patients with HEA.

Applications

Computed tomography (CT) manifestations in patients with HEA have certain characteristics. Primary diagnosis can be obtained by combining CT findings with clinical data, but pathological examination is still needed for a definite diagnosis.

Peer review

In this manuscript, the authors explore and analyze CT manifestations of five patients with HEA who were diagnosed definitely by pathological examination and immunohistochemistry, and provide reference for improving accurate rate of diagnosis. The manuscript is very well written.

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Analysis of prognostic factors and outcomes of gastric cancer in younger patients: A case control study using propensity score methods

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Author contributions: Kim KH and Kim MC contributed equally to this work; Kim KH designed the report and wrote the manuscript; Kim KH and Kim YM acquired the data; Kim KH and Kim MC analyzed and interpreted the data; Kim MC and Jung GJ provided the final approval of the version to be published.

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Abstract

AIM: To understand the clinicopathological and prognostic features of gastric cancer in younger and older patients.

METHODS: Between January 2002 and December 2008, 1667 patients underwent curative gastric surgery. For comparative purposes, the patients were divided into two groups: younger patients who were less than 40 years old (112 patients), and older patients who were 40 years old and older (1555 patients). In both groups, propensity scoring methods were used to select patients with similar disease statuses. A total of 224 matched cases, with 112 patients in each group, were included in the final analysis.

RESULTS: Compared to the older group, the younger group with gastric cancer had a significantly higher percentage of females ($P = 0.007$), poorly differentiated

or signet ring cell carcinoma ($P < 0.001$), advanced T stage gastric cancer ($P = 0.045$), and advanced tumor-node-metastasis stage cancer ($P = 0.036$). The older group with gastric cancer had more comorbidities ($P < 0.001$). With the exception of the number of lymph node dissection ($P < 0.001$) and retrieved lymph node ($P = 0.010$), there were no statistically significant differences between the postoperative outcomes of the two groups. During the follow-up period, there were 19 recurrences in the younger group and 11 recurrences in the older group. The overall five-year survival rates in the younger and older groups were 84.3% and 89.6%, respectively ($P = 0.172$). There were no significant differences ($P = 0.238$) in the overall survival of patients with advanced T stage gastric cancer in the two groups, with five-year survival rates of 70.8% in the younger group and 79.5% in the older group. With regard to the age-adjusted survival rate, there was significant difference between the two groups ($P = 0.225$).

CONCLUSION: In spite of aggressive cancer patterns in the younger group with gastric cancer, the younger group did not have a worse prognosis than the older group in our study.

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Key words: Gastric cancer; Younger patients; Prognosis

Core tip: In this study, propensity scoring methods were used to select patients with similar disease statuses. A total of 224 matched cases (112 patients in each group) were included in the analysis. The younger group with gastric cancer had more aggressive patterns than did the older group. The overall five-year survival rates between the younger and older groups were not significantly different. While there were more cases of aggressive cancer patterns in the younger group, early

diagnosis and curative resections improved the prognosis and patient survival; the younger group with gastric cancer did not show a worse prognosis than the older group.

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INTRODUCTION

Gastric cancer is one of the most common digestive cancers in the world. In addition, gastric cancer is the fourth most common cancer, and the second leading cause of cancer-related death, with approximately 700000 deaths annually^[1]. The prevalence of gastric cancer remains high in some Asian countries, especially in South Korea and Japan. While the incidence of advanced gastric cancer is decreasing in developed countries as a result of recent developments in medical screening, routine screening does not include people younger than 40 years of age, so gastric cancer in younger patients is a disturbing problem. In addition, gastric cancer is difficult to detect in younger patients who are asymptomatic, even in the advanced stages. The proportion of patients with gastric cancer ranges from 6% to 15% in patients younger than 41 years of age^[2-4]. There is controversy about whether gastric cancer differs between younger and older patients. Several reports have suggested that younger patients are often diagnosed with advanced-stage disease, and younger patients have been observed to have a markedly worse prognosis than their older counterparts^[5-10]. However, another recent report showed the prognosis for younger patients with gastric cancer to be equal or better than that of older patients^[11-17].

The proportion of younger patients with gastric cancer is smaller than that of older patients. Therefore, most studies have used small cases series of younger patients and large cases series of older patients for their comparisons^[2-15]. In this case-control study, matching was performed with the aim of selecting subsets of case and control groups with similar distributions of observed covariates (operation date and type of gastrectomy), thereby increasing the robustness of this retrospective observational study by reducing the bias that can be introduced by unbalanced groups. We used propensity scoring methods to evaluate whether the prognosis for younger patients with gastric cancer is equal to or poorer than that of older patients.

MATERIALS AND METHODS

Patients and data collection

From a prospectively collected gastric cancer database,

we identified 1945 patients who underwent gastric surgery between January 2002 and December 2008. Among them, 1667 patients who had no peritoneal seeding or distant liver metastasis underwent R0 resection. We defined the younger group with gastric cancer as being less than 40 years old, which is similar to the cut-off that has been used in previous reports^[3,5-6,13-15,17-19]. We divided the 1667 gastric cancer patients into two groups: younger patients who were less than 40 years old (112 patients), and older patients who were at least 40 years old (1555 patients). To reduce potential confounders in this retrospective observational study, the values of the propensity scores were used to adjust for differences between the two groups with regard to operation date and type of gastrectomy. A total of 224 matched cases, with 112 patients in each group, were included in the final analysis. The data were prospectively retrieved from operative and pathological reports, and follow-up data were obtained from the outpatient clinical database. Clinicopathological characteristics, postoperative outcomes, postoperative morbidities and mortalities, and survival rates were retrospectively compared between the two groups. The following data were obtained for each patient: age, gender, body mass index (BMI), comorbid disease, tumor size, histologic type, tumor location, tumor-node-metastasis (TNM) stage, and postoperative outcomes. Postoperative outcomes included operative time, hospital stay, type of gastrectomy, reconstruction, extent of lymph node dissection, number of retrieved lymph nodes, recurrence, and survival.

In this study, the gastric cancer stage was classified according to the 7th edition of the American Joint Committee on Cancer staging criteria^[20]. Standard lymph node dissection (D2) was performed according to the 2010 Japanese gastric cancer treatment guidelines^[21].

Adjuvant chemotherapy, follow-up protocol, and recurrence

Adjuvant chemotherapy was performed in patients with pathologically identified advanced gastric cancer who had provided their informed consent. Adjuvant chemotherapy was not performed for patients with stage I gastric cancer, but adjuvant chemotherapy was performed for patients within stage II A or higher gastric cancer. Follow-up results were obtained from patient hospital records and telephone calls, and recurrence was determined by endoscopy, computed tomography, and positron emission tomography. All patients who received follow-up were monitored postoperatively with routine blood tests, tumor markers (carcinoembryonic antigen and carbohydrate antigen 19-9), chest radiography, endoscopy, and computed tomography. In patients with early gastric cancer, follow-up studies were performed every six months for two years and annually for three years. For patients with advanced gastric cancer, follow-up studies were performed every three months for the first year, every six months for the second year, and annually for following three years.

We classified recurrence patterns into four catego-

Table 1 Clinicopathological features of the patients

Clinicopathological feature	Younger patients (<i>n</i> = 112)	Older patients (<i>n</i> = 112)	<i>P</i> -value
Gender			0.007
Male	49	70	
Female	63	42	
BMI (mean ± SD, kg/m ²)	22.4 ± 3.4	23.1 ± 2.7	0.086
Comorbidity			< 0.001
No	100	74	
Yes	12	38	
Size of main lesion (mean ± SD, mm)	4.1 ± 3.1	3.9 ± 3.3	0.604
Histologic type			< 0.001
Well differentiated	7	29	
Moderate differentiated	19	34	
Poorly differentiated	70	41	
Signet ring cell	14	5	
Other	2	3	
Lauren classification			< 0.001
Intestinal	18	58	
Diffuse	72	34	
Mixed	22	20	
Tumor location			0.154
Upper	15	15	
Middle	40	25	
Lower	55	70	
Whole	2	2	
T stage ¹			0.045
EGC	50	66	
AGC	62	46	
N stage ¹			0.132
N0	67	72	
N1	12	16	
N2	11	14	
N3	22	10	
Stage ¹			0.036
I	57	75	
II	21	11	
III	34	26	

¹Based on the 7th edition of the American Joint Committee on Cancer classification.

ries^[22]: locoregional, hematogenous, peritoneal, and distant lymph nodes. Locoregional recurrence was defined as the presence of tumors in the adjacent organs, which includes gastric bed, anastomosis, gastric stump, and regional lymph nodes. Hematogenous recurrence included recurrence in the liver, lung, bone, brain, or other distant sites. Peritoneal recurrence was defined as peritoneal seeding or ovarian metastases (Krukenberg's tumor). Recurrence in distant lymph nodes was defined as extraabdominal lymph nodes.

Statistical analysis

To reduce bias, a propensity scoring approach was used to match the younger and older patients according to the operation date and type of gastrectomy. Patients less than 40 years of age (case) were matched to patients at least 40 years old (control) by using patient identifiers and operation dates (± 15 d). Matching was performed to select subsets of case and control groups with similar distributions of the observed covariates operation date and type of gastrectomy. The matching increased the robustness

of the retrospective observational design by reducing the bias that can be introduced from unbalanced groups. The data were summarized using frequencies and percentages for categorical variables and means and standard deviations for continuous variables. After descriptive analyses were performed, a Fisher's test was used to compare categorical variables between the groups, and a Mann-Whitney *U* test was used to compare continuous variables between the groups. *P* values of less than 0.05 were considered to be statistically significant. Survival curves were calculated using the Kaplan-Meier method. All statistical analyses were conducted using SPSS version 18.0 (SPSS, Chicago, IL).

RESULTS

Clinicopathological features between younger and older patients with gastric cancer

The proportion of females in the younger group was greater than that in the older group (*P* = 0.007). The older group had more comorbidities than did the younger group (*P* < 0.001), and the younger group had more poorly differentiated or signet ring cell gastric carcinomas (*P* < 0.001). In addition, using the Lauren classification, a diffuse form of cancer was found in 64.3% of patients in the younger group (*P* < 0.001). Compared to the older group, the younger group had more aggressive T stage gastric cancer (*P* = 0.045) and more cases of advanced TNM stage cancer (*P* = 0.036). There were no significant differences in the BMI, tumor size, tumor location, and N stage between the two groups (Table 1).

Postoperative outcomes and complications

Table 2 shows the postoperative outcomes of the two groups; there were statistically significant differences in the extent of lymph node dissection (*P* < 0.001) and the number of retrieved lymph nodes (*P* = 0.010). There were no significant differences in operative time, hospital stay, operative method, type of gastrectomy, type of anastomosis, or cancer-related organ resection. Complications in the younger group were found in 10 (8.9%) of the 112 patients, and one (0.9%) major complication required endoscopic control of intraluminal bleeding. Nine minor complications in the younger group were treated with conservative management. In the older patients, there were 16 (14.3%) complications, 3 (2.7%) of which were major; one patient required endoscopic control of intraluminal bleeding, and two patients required re-operation to repair duodenal stump leakage. Other complications in older patients were also treated with conservative management. There were no statistically significant differences between the two groups in postoperative complications (*P* = 0.297).

Tumor recurrence and survival

The median follow-up period was 79.2 mo in the younger group and 80.3 mo in the older group. We performed adjuvant chemotherapy for patients within stage II or

Table 2 Postoperative outcomes

Postoperative outcomes	Younger patients (n = 112)	Older patients (n = 112)	P-value
Operative time (min, mean ± SD)	207.3 ± 57.1	205.7 ± 56.7	0.832
Hospital stay (d, mean ± SD)	7.9 ± 2.6	10.1 ± 13.0	0.087
Operative method			0.268
Laparoscopy	46	37	
Open	66	75	
Type of gastrectomy			1.000
Total	28	28	
Subtotal	84	84	
Type of anastomosis			0.856
B-I	48	52	
B-II	35	32	
R-Y	29	28	
Lymph node dissection			< 0.001
D2	43	79	
Over D2	69	33	
Number of retrieved lymph nodes (mean ± SD)	41.4 ± 16.4	36.3 ± 13.2	0.010
Cancer-related combined resection			0.409
No	107	103	
Yes (number of patients)	5	9	
Spleen ¹	4	6	
Pancreas ¹	0	3	
Liver ¹	0	1	
Adrenal gland ¹	0	1	
Small bowel ¹	1	0	
Ovary ¹	1	0	
Median follow-up duration (mo, range)	79.2 (8.0-137.7)	80.3 (0.7-137.5)	0.523
Postoperative complications			0.297
No	102	96	
Yes	10	16	
Wound problem	3	5	
Intra-abdominal bleeding	1	1	
Intra-luminal bleeding	3 (1 ²)	4 (1 ²)	
Ileus	1	1	
Duodenal stump leakage	0	2 ²	
Acute pancreatitis	0	1	
Pulmonary disease	0	2	
Hepatic disease	1	0	
Dumping syndrome	1	0	

¹The number of resected organs is a combined number; ²Intra-luminal bleeding was treated endoscopically; duodenal stump leakage required reoperation.

higher. In the 55 patients in the younger group with stage II or higher cancer, 49 patients received with adjuvant chemotherapy; in the 37 older patients with stage II or higher, 36 patients adjuvant chemotherapy. There was no significant difference in the use of adjuvant chemotherapy between the two groups ($P = 0.235$). During the median follow-up period, tumor recurrences occurred in 19 cases in the younger group and 11 cases in the older group. The median time to recurrence after surgery was 17.8 mo in the younger group and 18.5 mo in the older group, but the difference between the groups was not statistically significant ($P = 0.169$). In the younger group, peritoneal recurrence was the most common recurrence pattern. However, in the older group, the most common recurrences were locoregional and hematogenous (Table 3). The overall five-year survival rates in the younger and

Table 3 Tumor recurrence and patterns

Postoperative outcomes	Younger patients (n = 112)	Older patients (n = 112)	P-value
Median follow-up duration (mo, range)	79.2 (8.0-137.7)	80.3 (0.7-137.5)	0.523
Chemotherapy (stage II or higher) n (%)	49/55 (89.1)	36/37 (97.3)	0.235
Tumor recurrence			0.169
No	93	101	
Yes	19	11	
Time to recurrence after surgery (mo, mean ± SD)	17.8 ± 9.8	18.5 ± 12.6	0.862
Recurrence patterns ¹			
Locoregional	6	5	
Hematogenous	6	5	
Peritoneal	10	3	
Distant lymph nodes	2	2	

¹Combined numbers.

older groups were 84.3% and 89.6%, respectively. There were no significant differences in the survival rates between the two groups ($P = 0.172$). In cases of advanced T stage gastric cancer, the overall five-year survival rates were 70.8% in the younger group and 79.5% in the older group ($P = 0.238$) (Figure 1). There was no significant difference in the age-adjusted survival rate between the two groups ($P = 0.225$) (Figure 2).

DISCUSSION

Gastric cancer is generally considered to be an age-related disease, and more than half of gastric cancer patients are older than 60 years of age. Gastric cancer rates have been consistently decreasing as a result of recent medical screening systems. However, gastric cancer in younger group populations remains a serious problem in some countries, such as South Korea, where routine screening does not occur in people under 40 years of age. Therefore, in the absence of gastrointestinal symptoms, gastric cancer is difficult to diagnoses in young people, even in the advanced stages of the disease. This retrospective matched case-control study was performed to determine the clinicopathological and prognostic features of gastric cancer in younger and older patients.

Studies have generally been limited by a small number of patients, the inclusion of historical data, a lack of comparison with similar control groups, and a limited ability to account for disease survival. Thus, most studies have made comparisons between small cases series of younger patients and large cases series of older patients with gastric cancer^[2-15]. These unbalanced comparisons may generate biased and inconsistent results. Therefore, in this study, we matched the younger and older groups with increasing robustness to reduce the bias that can be caused by unbalanced groups.

Many studies have shown interesting clinical differences between younger and older patients with gastric cancer. Most notably, the gender ratio is different be-

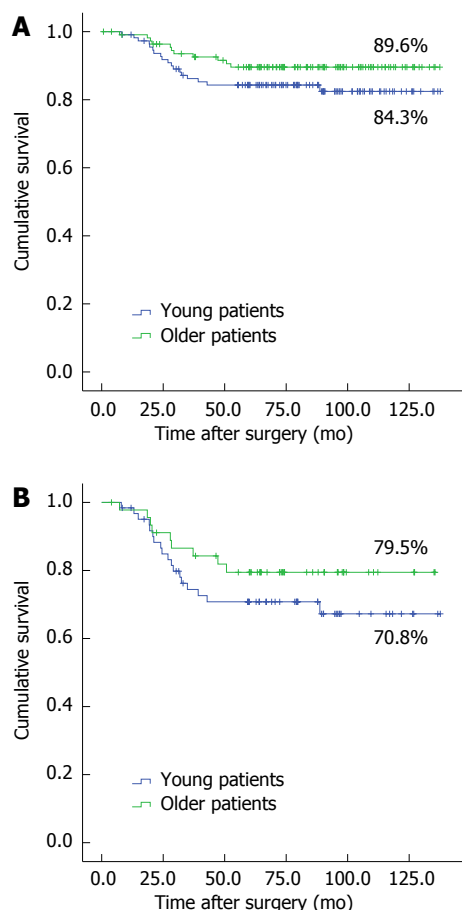


Figure 1 Comparison of overall five-years survival rates in the younger and older groups of patients with gastric cancer. A: There were no significant differences between the two groups ($P = 0.172$); B: In cases of advanced T stage gastric cancer, there were also no significant differences in the overall five-year survival rates ($P = 0.238$).

tween younger and older patients with gastric cancer, which means that gender-associated differences can be amplified in different age group. For example, younger groups are comprised of more female patients than older groups^[4,5,11,12,14,15], and in male patients, malignant neoplasms often occur in the stomach, esophagus, liver, colon, or rectum^[23]. There is currently no widely accepted explanation for the reversal of the male/female ratio in younger patients with gastric cancer. Several reports have suggested that the predominance of females may be caused by hormonal factors, such as the amount of estrogen and the higher percentage of estrogen receptor-positive cells in younger females^[15,24]. However, the relationship between hormones and the prognosis of gastric cancer remains controversial. In our study, there was a significantly higher proportion of females in the younger group compared to the older group ($P = 0.007$).

Another interesting clinical difference between younger and older patients with gastric cancer is the histologic aggressiveness of the cancer. Several studies have reported similar clinicopathological features in younger patients with gastric cancer compared to older patients^[2-6,11-14,23]. While there are some differences among the reports,

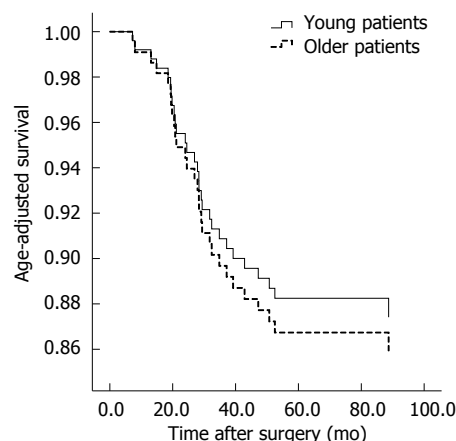


Figure 2 Age-adjusted survival curves. There were no significant differences between the two groups ($P = 0.225$).

younger patients with gastric cancer generally have more diffuse gastric cancer than older patients (following the Lauren classification); younger patients also tend to have a more poorly differentiated carcinoma or signet ring cell carcinoma, a more advanced T stage, a greater likelihood to have lymph node metastasis, and a greater proportion of tumors in the body of the stomach. These features include poorly differentiated diffuse adenocarcinomas, which are associated with genetic abnormalities^[25-27]. Genetic susceptibility is one major factor that is associated with the development of gastric cancer. Diffuse types of gastric cancer are reported to be common in younger groups that have a genetic predispositions, and these diffuse gastric cancers lead to a poorer prognosis, the intestinal type of gastric cancer, which is associated with a better prognosis than diffuse types, is more common in older patients^[28,29]. In younger compared to older patients, gastric cancer is significantly more likely to be located in the body of the stomach^[17]. In the present study, poorly differentiated and signet ring cell type cancers were more predominant in the younger group than in the older group ($P < 0.001$). Following the Lauren classification, the diffuse type was more prevalent in the younger group ($P < 0.001$). Although there were no significant differences in tumor location, there was a higher frequency of tumors in the middle portion of the stomach in the younger group compared to the older group. Tumor staging is a crucial factor for determining whether and how to perform surgery and tumor stage correlates closely with prognosis. As already noted, the younger group with gastric cancer had more aggressive T and N stages than did the older group. Although there were significant differences in the N stage, the T and TNM stages were more advanced in the younger group compared to the older group ($P = 0.045$ and $P = 0.036$, respectively).

The prognosis of young patients with gastric cancer has been debated for years. Compared to older patients, younger patients have more aggressive patterns of gastric cancer, and several reports have shown that younger patients with gastric cancer have worse prognoses than old-

er patients^[5-10]. However, another recent report showed the prognosis in the younger group to be equal to or better than that of the older group^[11-17]. The good prognosis and improved survival rate that have recently been found in younger patients may be a result of improved surgical techniques and extended lymph node dissections for gastric cancers with aggressive patterns of the disease. In our study, more extensive lymph node dissections (over D2) were performed in the younger group with gastric cancer ($P < 0.001$), and more lymph nodes were retrieved in the younger group than in the older group. The overall five-year survival rates in the younger and older groups were 84.3% and 89.6%, respectively, but the difference was not statistically significant ($P = 0.172$). For advanced T stage gastric cancer, the overall five-year survival rates were 70.8% in the younger group and 79.5% in the older group, and the difference was also not statistically significant ($P = 0.238$). After adjusting for age, there was no significant differences in the survival curve between the two groups ($P = 0.225$) (Figure 2).

In our study, tumor recurrence occurred in 19 cases in the younger group and 11 cases in the older group. Kong *et al*^[17] reported that peritoneal metastasis occurred more often in younger patients than older patients with gastric cancer. Peritoneal metastasis is predominantly seen in gastric cancer with ascites, which is often poorly differentiated. The link to poorly differentiated gastric cancer may partially explain why peritoneal metastasis is more prevalent in younger patients. While there was no significant difference in tumor recurrence between the two groups ($P = 0.169$), there were more peritoneal metastases in the younger group than in the older group.

The lower comorbidity and postoperative complication rates are advantages for young patients with gastric cancer. Comorbidity is an important factor that affects postoperative complications^[30]. Yoo *et al*^[31] reported that the presence of postoperative complications such as leakage, are negative prognostic factors in patients with advanced gastric cancer. Therefore, in our study, a lower comorbidity and a lack of major postoperative complications such as leakage, may have improved the prognosis in the younger group with gastric cancer, which may have counteracted the aggressive patterns of gastric cancer that were present in the younger group.

A delay in gastric cancer diagnosis may be a factor in the poor prognosis that is observed in younger groups with gastric cancer. If a younger patient has a suspicious lesion or a familial history of gastric cancer, physicians should carefully observe this patient and perform regular endoscopic evaluations. Early diagnosis of younger patients with gastric cancer will improve their surgical prognosis.

In our case-control matched study, the younger group with gastric cancer had more advanced and aggressive patterns, which is consistent with previous studies. While the younger group with gastric cancer tended to have aggressive patterns, early diagnosis and curative resection improved the prognosis of the younger group with gastric cancer.

COMMENTS

Background

There is controversial about whether younger patients with gastric cancer have a worse prognosis than older patients. The proportion of younger patients with gastric cancer is smaller than that of older patients. Therefore, most studies have used small case series of younger patients and large cases series of older patients for their comparisons.

Research frontiers

In this case-control study, matching was performed to select subsets of case and control groups with similar distributions of the observed covariates operation date and type of gastrectomy, which increased the robustness of the retrospective observational study by reducing the bias that can be introduced by unbalanced groups.

Innovations and breakthroughs

In this matched case-control study, the authors used propensity scoring methods to evaluate whether the prognosis for younger patients with gastric cancer is equal to or poorer than that of older patients.

Applications

While there were more cases of aggressive cancer patterns in the younger group with gastric cancer, early diagnosis and curative resection improved the prognosis and patient survival.

Peer review

This study aimed at analyse the prognostic outcomes of gastric cancer in younger patients compared to older subject using propensity score methods. The manuscript is well written and the results are interesting.

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***Helicobacter heilmannii* sensu stricto-related gastric ulcers: A case report**

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Author contributions: Matsumoto T designed and conducted research, wrote the manuscript and had primary responsibility for final content; Kawakubo M, Kubota S and Sugano M performed the research; Ogiwara N performed transmission electron microscopy analysis; Akamatsu T and Koide N performed endoscopic procedures and treatment; Kawakami Y proofread and revised the manuscript; Katsuyama T made the pathological diagnosis, and Ota H designed research, made the pathological diagnosis and wrote the manuscript.

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without corpus atrophy. Urea breath test and *H. pylori* culture were negative, but Giemsa staining of biopsies revealed tightly coiled bacteria that immunostained with anti-*H. pylori* antibody. Sequencing of SH9 16S rRNA and the partial urease A and B subunit genes showed that the former sequence had highest similarity (99%; 1302/1315 bp) to *Helicobacter heilmannii* (*H. heilmannii*) sensu stricto (*H. heilmannii* s.s.) BC1 obtained from a bobcat, while the latter sequence confirmed highest similarity (98.3%; 1467/1493 bp) to *H. heilmannii* s.s. HU2 obtained from a human. The patient was diagnosed with multiple gastric ulcers associated with *H. heilmannii* s.s. infection. After triple therapy (amoxicillin, clarithromycin, and lansoprazole) with regimen for eradicating *H. pylori*, gastroscopy showed ulcer improvement and no *H. heilmannii* s.s. upon biopsy.

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Key words: *Helicobacter heilmannii* sensu stricto; "*Helicobacter heilmannii*" type 2; Gastric ulcers; Urease gene; Non-*Helicobacter pylori* helicobacters

Core tip: Herein we report a case of *Helicobacter heilmannii* sensu stricto (*H. heilmannii* s.s.)-related gastric ulcers. The organism was identified as *H. heilmannii* s.s. which is a recently identified species of the former "*H. heilmannii*" type 2 group by transmission electron microscopy, 16S rRNA gene and the partial urease gene sequences. After triple therapy with regimen for eradicating *Helicobacter pylori*, gastroscopy showed ulcer improvement and no *H. heilmannii* s.s. upon biopsy.

Abstract

A spiral bacterium (SH9), morphologically different from *Helicobacter pylori* (*H. pylori*), was found in a 62-year-old woman's gastric mucosa. Gastroscopic examination revealed multiple gastric ulcers near the pyloric ring; mapping gastric biopsy showed mild mononuclear infiltration with large lymphoid follicles in the antrum,

Matsumoto T, Kawakubo M, Akamatsu T, Koide N, Ogiwara N, Kubota S, Sugano M, Kawakami Y, Katsuyama T, Ota H. *Helicobacter heilmannii* sensu stricto-related gastric ulcers: A case report. *World J Gastroenterol* 2014; 20(12): 3376-3382 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/>

INTRODUCTION

Helicobacter spp. have been isolated from an ever-expanding range of host species^[1].

Although gastric non-*Helicobacter pylori* (*H. pylori*) helicobacters (NHPH)^[2-5] are ubiquitous in animals, including primates, dogs, cats and pigs, their occurrence in humans is rather low compared to the occurrence of *H. pylori*. NHPH infection in human gastric mucosa has been suggested to occur through zoonotic transmission^[6]. Sequence analysis of 16S rRNA genes detected in NHPH-positive gastric biopsies revealed the presence of 2 sequence types. This has led to the sub-classification of gastric NHPH as “*Helicobacter heilmannii* (*H. heilmannii*)” type 1 and “*H. heilmannii*” type 2. “*H. heilmannii*” type 1 is identical to *Helicobacter suis*, which colonizes the stomachs of pigs^[7]. “*H. heilmannii*” type 2 represents a group of species, including *Helicobacter felis*, *Helicobacter bizzozeronii*, *Helicobacter salomonis*, and the previously named “*Candidatus H. heilmannii*” or “*H. heilmannii*”^[8].

Furthermore, to avoid confusion with the name *H. heilmannii*, Haesebrouck *et al*^[9] have proposed that *H. heilmannii* sensu lato (*H. heilmannii* s.l.) be used to refer to the whole group of non-*H. pylori* helicobacters detected in the human or animal stomach, for instance, when only histopathology, electron microscopy, or crude taxonomic data are available. The name *H. heilmannii* sensu stricto (*H. heilmannii* s.s.) or other species names should be used whenever bacteria are really identified to the species level.

In line with this concept, Smet *et al*^[10] reported the successful isolation of *H. heilmannii* s.s. (formerly “*Candidatus H. heilmannii*”) from the gastric mucosa of cats. The bacterium was commonly found in feline rather than canine hosts, and sequence analysis of the urease gene of the bacterium showed high similarity to that of the originally named “*Candidatus H. heilmannii*”^[10,11].

To date, few studies have identified gastric NHPH to the species level by genetic analysis^[5,11-14]. Thus far, the differences in the pathogenicity and eradication therapy response between *Helicobacter suis* (*H. suis*) and *H. heilmannii* s.s. infection in humans have not been understood.

We describe here the case of a patient with multiple gastric ulcers infected with *H. heilmannii* s.s., as defined in a report by Smet *et al*^[10].

CASE REPORT

A 62-year-old woman underwent gastrointestinal endoscopy because of the sudden onset of epigastralgia and multiple gastric ulcers were found near the pyloric ring (Figure 1). Gastric tissue biopsies were taken from the ulcer edge and revealed regenerative changes with active inflammation. For the treatment of gastric ulcers, lansoprazole (30 mg/d) was administered once daily to the patient for 4 wk, which resulted in the resolution of

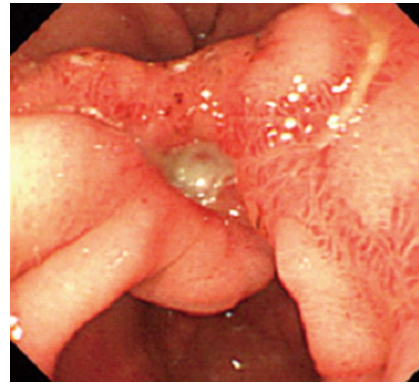


Figure 1 Initial gastrointestinal endoscopy shows gastric ulcers in the antrum.

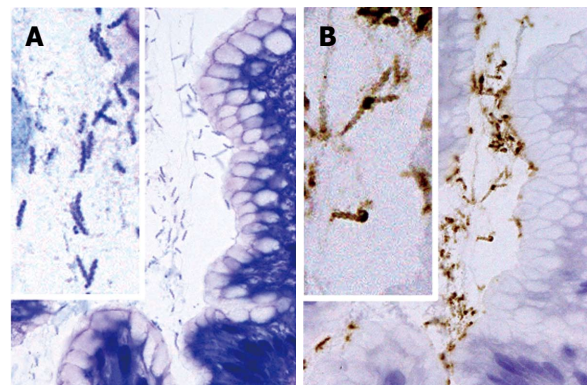


Figure 2 Histological findings in non-*Helicobacter pylori* helicobacter SH9 in gastric mucosa. The organisms are present in small groups in gastric pits (A, B). The organisms display a tightly coiled spiral configuration and (B) are positive for the anti-*Helicobacter pylori* (*H. pylori*) antibody. (A) HE, original magnification, $\times 1000$; (B) Immunostaining with anti-*H. pylori* antibody, original magnification, $\times 1000$.

her symptoms. Gastrointestinal endoscopic examination after lansoprazole therapy revealed multiple gastric scars near the pyloric ring. Gastric tissue biopsies were taken from the scars and revealed the regenerative changes with active inflammation and the presence of *H. heilmannii* s.l. on hematoxylin and eosin (H and E) and Giemsa staining in the mucus covering the gastric mucosa; these bacteria were larger and more tightly coiled than *H. pylori* (Figure 2A). The spiral bacteria were immunostained with a rabbit polyclonal antibody against *H. pylori* (Dako, Carpinteria, CA) (Figure 2B); this antibody is generated using the whole *H. pylori* bacterial extract as the antigen, and it exhibits cross-reactivity with “*Candidatus H. heilmannii*”^[15] and *H. suis*^[16]. The cross-reactivity of anti-*H. pylori* antibodies from different sources with “*Candidatus H. heilmannii*”^[17] and *H. suis*^[18] have been reported to be similar.

The patient was referred to our hospital for verification of the diagnosis of *H. heilmannii* s.l.. The patient’s laboratory data were within the normal range. Serum anti-*H. pylori* immunoglobulin G antibody, measured with an enzyme-linked immunosorbent assay (ELISA) kit using the E plate test (Eiken Kagaku, Inc., Tokyo, Japan), was 2.1 IU/mL (cut-off value: 10 U/mL). The ¹³C-urea

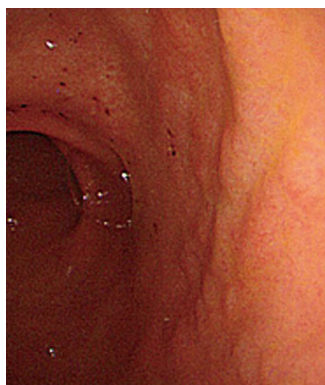


Figure 3 Gastrointestinal endoscopy after lansoprazole therapy shows erosion and a slightly coarse granular appearance in the antrum.

breath test result (UBiT, Otsuka Pharmaceutical Co., Tokushima, Japan) was negative. The patient was not administered any non-steroidal anti-inflammatory drugs. The patient had 3 household cats.

Gastrointestinal endoscopy and mapping gastric biopsy according to the updated Sydney system^[19] was performed in our hospital for the histological evaluation of gastritis. In addition, antral biopsy specimens were taken for the culture of *H. pylori* and for the inoculation of mice by peroral administration of gastric mucosal homogenates. On gastrointestinal endoscopic examination in our hospital, background gastric mucosa of the present patient showed erosive and a slightly coarse appearance in the antrum (Figure 3), and normal appearing mucosa, without atrophy, in the corpus. Histopathological examination of gastric biopsy specimens showed mild mononuclear infiltration with large lymphoid follicles, and without neutrophil infiltration and epithelial degeneration in the antrum (Figure 4) and normal morphology in the corpus. Tightly coiled spiral bacteria were identified in the antrum, but not in the corpus, by histological examination; however, *H. pylori* was not isolated by microbiological culture. Based on these clinical, endoscopic, and histological findings, a diagnosis of multiple gastric ulcers associated with *H. heilmannii* s.l. (designated as SH9) infection was made.

After confirmation of the *H. heilmannii* s.l. infection, the patient was treated with 1-wk triple therapy consisting of lansoprazole (30 mg/d), amoxicillin (1500 mg/d), and clarithromycin (1200 mg/d) according to the standard protocol for eradicating *H. pylori* infection. Twelve wks after the cessation of treatment, gastrointestinal endoscopy showed gastric scars. Further, gastric biopsy mapping showed smaller lymphoid follicles in a biopsy obtained from the greater curvature of the antrum as well as the normal appearance of the corpus, without the presence of these organisms, as evaluated by histology and immunostaining with anti-*H. pylori* antibody. The patient showed complete remission at the follow-up endoscopy performed 7 years after the completion of eradication therapy, and currently remains healthy.

An attempt was made to maintain and propagate SH9 *in vivo* by inoculating homogenized whole-antral

biopsy specimens from the patient into the stomachs of specific pathogen-free mice^[20,21]. Transmission electron microscopy of the mouse gastric mucosa colonized with SH9 revealed that the bacteria showed 5-9 coils per cell with flagella at each pole, and were 4-6 μm long and 1 μm wide, which is larger and more coiled than *H. pylori* (Figure 5). In order to characterize SH9, the 16S rRNA and urease genes were sequenced. Polymerase chain reaction (PCR) amplification of the 16S rRNA gene from SH9-derived DNA isolated from the mouse was performed using 2 sets of primers: 79F (5'-GTGAGTAATGCATAGATGACATGCCC, originally designed in this study) and H679R (5'-ATTCCACCTACCTCTCCCA), and H279F (5'-CTATGACGGGTATCCGGC) and 1494R (5'-TACGGCTACCTTGTACGAC)^[22-24]. The urease gene was amplified by PCR using the primers U430F (5'-GCKGAWTTGATGCAAGAAGG)^[11] and UH6R2 (5'-TTATCCAAGTGGTGGCACACC)^[13] and sequenced. The sequence obtained represented the partial 16S rRNA gene with a readable sequence of 1323 bp (GenBank/EMBL/DDBJ accession no. AB778508) determined from the DNA. The closest match of the 16S rRNA sequence with *H. heilmannii* s.s. BC1 obtained from a bobcat was obtained with 99% (1302/1315 bp) identity (GenBank/EMBL/DDBJ accession no. AF506772). The urease gene from SH9 was amplified by PCR using the primer U430F together with H6-R2. The sequencing represented the partial urease gene with a readable sequence of 1493 bp (GenBank/EMBL/DDBJ accession no. AB778507). The comparatively high similarities of the DNA sequence to that of *H. heilmannii* s.s. HU2 (AF508012) obtained from a human, BC1 (AF507996), ASB2 (HM625825) isolated from an European feline, and ASB1 (HM625826) isolated from an European feline were 98.3% (1467/1493 bp), 98.3% (1427/1451 bp), 90.5% (964/1065 bp), and 90.4% (958/1060 bp), respectively. The DNA sequences of the urease genes from SH9 and from reference organisms were aligned using GENETYX ver. 9 (GENETYX Corp., Tokyo, Japan), and the resulting alignment was modified to remove regions containing gaps. A phylogenetic tree was constructed according to the neighbor-joining method^[25,26] and the Kimura model. The stabilities of the relationships were assessed by bootstrap analysis comprising 1000 data settings. Figure 6 demonstrates a phylogenetic tree constructed on the basis of urease gene analysis, and grouping of clusters as shown by O'Rourke *et al.*^[11]. The results demonstrated high levels of homology with cluster B.

DISCUSSION

We investigated the *H. heilmannii* s.s., designated as SH9, of human origin, both histologically and genetically by analysis of the sequences of both the 16S rRNA and urease genes.

We were unable to detect *H. pylori*, but detected a population of corkscrew-shaped organisms in gastric tissue biopsy samples taken from the marginal region of the gastric ulcers and from the antrum. The organisms were

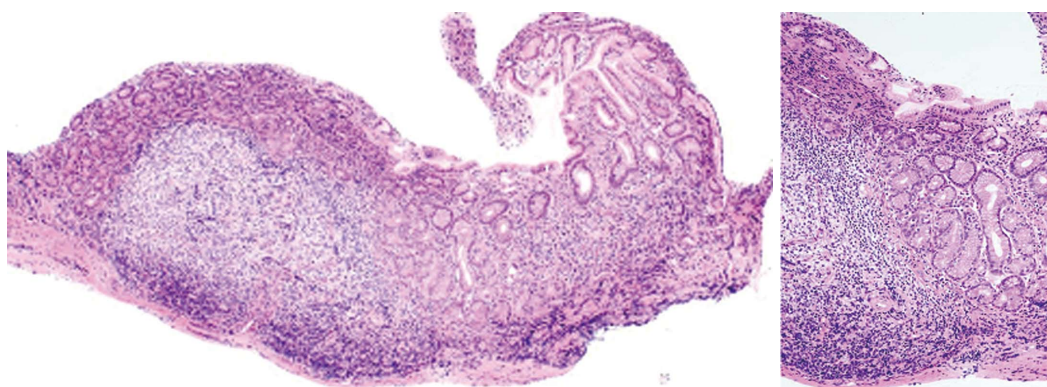


Figure 4 Histological findings of the lesser curvature of gastric antrum from a SH9 (*Helicobacter heilmannii sensu stricto*)-infected patient after the administration of lansoprazole for the treatment of gastric ulcers. The gastric mucosa shows large lymphoid follicles with germinal centers without epithelial degeneration.

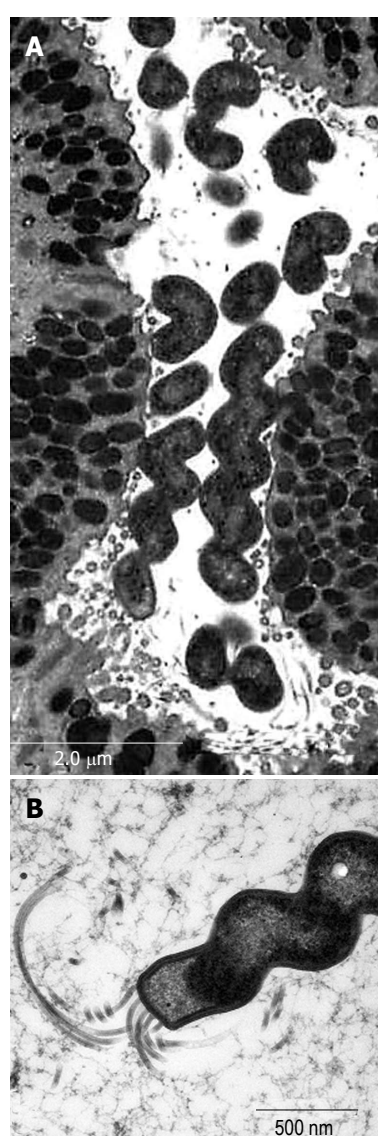


Figure 5 Ultrastructure of SH9 (*Helicobacter heilmannii* s.s.) infected mouse gastric mucosa. The organisms showing tightly coiled spiral configuration (A) with flagella (B) are positioned on the apical surface of the surface mucous cells.

found in the mucous gel or on the surface of mucous cells without adhesion to gastric epithelial cells. Back-

ground gastric mucosa of the patient showed chronic gastritis with prominent lymphoid follicles without atrophy, although the mapping biopsy was performed after administration of lansoprazole for the treatment of gastric ulcers. These findings are similar to those obtained in previous studies of *H. heilmannii* s.l.^[15,27-29]. The negative antibody test and urea breath-test results of the present patient were also consistent with previous results of *H. heilmannii* s.l. infection^[15]. The sensitivity of urea breath-test for detection of *H. heilmannii* s.l. was reported to be low^[30] and in this study, urea breath-test was negative. A false-negative urea breath-test test may occur due to the lower activity of *H. heilmannii* s.l. urease, as suggested by previous studies^[30-32].

The 16S rRNA gene sequence of SH9 exhibited 99.1% (1302/1314 bp) similarity to *H. heilmannii* s.s. BC1; this was higher than that to *H. suis*. In the subsequent comparison of the urease gene sequence, SH9 revealed apparently higher levels of urease gene sequence similarities to *H. heilmannii* s.s. HU2 and *H. heilmannii* s.s. BC1. In the phylogenetic analysis following sequencing of the urease genes, SH9 was classified as belonging to Cluster B, proposed by O'Rourke *et al*^[11] (Figure 6). Consequently, we identified SH9 as *H. heilmannii* s.s.. Interestingly, homologies to the urease gene sequences of *H. heilmannii* s.s. ASB1 and ASB2 isolated from European felines were approximately 90%. The slightly differences between the urease genes of SH9 and the European isolates may suggest geographical differences.

H. heilmannii s.l. infection have been reported in patients with acute^[29] and chronic gastritis^[15,27,28], duodenal ulcers^[33], gastric mucosa-associated lymphoid tissue (MALT) lymphoma^[15,27,34], or gastric carcinoma^[35]. However, since identification at the species level of *H. heilmannii* s.l. is often lacking, the pathogenicity of *H. suis* and *H. heilmannii* s.s. has not been clear. *H. suis* infection has, however, been reported to cause gastric MALT lymphoma in experimental animals^[16,18,36]. In contrast to *H. suis*, *H. heilmannii* s.s. infection in humans has rarely been reported. Interestingly, Dieterich *et al*^[12] reported multiple *H. heilmannii* s.l. strains infection in a gastric ulcer patient and in his 2 cats after partially analyzing the urease B gene and suggested transmission of *H. heilmannii* s.l. from

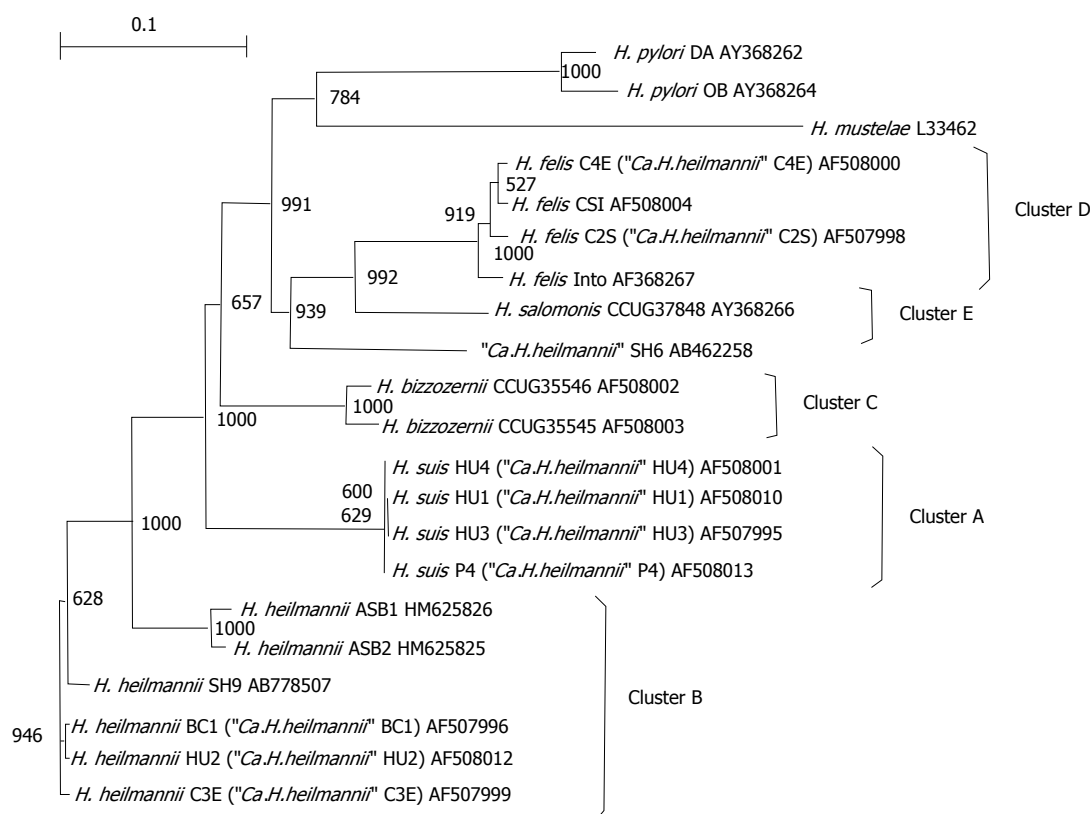


Figure 6 Phylogenetic tree based on partial *ureA* and *ureB* gene sequences, demonstrating the relationship between *Helicobacter* spp. from animals and humans. Clusters A through E correspond to *Helicobacter suis* (*H. suis*), *Helicobacter heilmannii* sensu stricto (*H. heilmannii* s.s.), *Helicobacter bizzozeronii* (*H. bizzozeronii*), *Helicobacter felis* (*H. felis*), and *Helicobacter salomonis* (*H. salomonis*), as described by O'Rourke *et al.*^[11]. *Ca.H.heilmannii*: *Candidatus H. heilmannii*; *H. pylori*: *Helicobacter pylori*.

the patient's household cats. Based on partial urease B gene sequences of *H. heilmannii* s.l. strains in their report, all of their *H. heilmannii* s.l. strains can be defined as *H. heilmannii* s.s. as the partial urease B gene sequences they reported demonstrated the highest similarity 98% with *H. heilmannii* s.s. (GenBank/EMBL/DDBJ accession no. L25079). In addition, the reported *H. heilmannii* s.s. isolates have commonly been observed in feline rather than canine hosts^[11]. The present patient also had 3 cats, again suggesting transmission of *H. heilmannii* s.s. from the patient's household cats.

H. heilmannii s.l. eradication by antimicrobial treatment regimens that are used for eradicating *H. pylori* results in clinical and histological improvement in *H. heilmannii* s.l.-associated gastroduodenal diseases^[15,29,33,34,37]. Furthermore, in the present case, 1-wk triple therapy (amoxicillin, clarithromycin, and lansoprazole) for the eradication of *H. pylori* was effective. Thus, the regimen for *H. pylori* eradication may also be effective for *H. heilmannii* s.s. PCR analysis is desirable for the precise evaluation of *H. heilmannii* s.s. infection status after eradication therapy. In the present case, *H. heilmannii* s.s. was considered to be successfully eradicated, because the patient showed complete remission at the follow-up endoscopy performed 7 years after the completion of eradication therapy. Currently, the patient is healthy.

In conclusion, we presented a case of *H. heilmannii*

s.s.-associated multiple gastric ulcers. Further studies are needed to clarify the pathogenicity of *H. heilmannii* s.s. and to verify effective eradication strategies for *H. heilmannii* s.s..

COMMENTS

Case characteristics

A 62-year-old female with *Helicobacter heilmannii* sensu stricto (*H. heilmannii* s.s.)-related gastric ulcers.

Clinical diagnosis

Sudden onset of epigastralgia.

Differential diagnosis

Gastroesophageal reflux disease, Gastric cancer, nonsteroidal anti-inflammatory drug-related gastric ulcer, *Helicobacter pylori* (*H. pylori*)-related gastric ulcer.

Laboratory diagnosis

Laboratory data were within the normal range. Serum anti-*H. pylori* immunoglobulin G antibody and the ¹³C-urea breath test result were negative.

Imaging diagnosis

Gastroscopic examination revealed multiple gastric ulcers near the pyloric ring.

Pathological diagnosis

Pathological and molecular biological examinations revealed the gastric ulcer with *H. heilmannii* s.s. infection.

Treatment

The patient was treated with 1-wk triple therapy consisting of lansoprazole (30 mg/d), amoxicillin (1500 mg/d), and clarithromycin (1200 mg/d) according to the standard protocol for eradicating *H. pylori* infection.

Related reports

H. heilmannii s.s. infection in humans is not very well reported.

Term explanation

The name *Helicobacter heilmannii* sensu stricto is *H. heilmannii* identified to the species level.

Experiences and lessons

H. heilmannii s.s. is also related to a human gastric ulcer.

Peer review

This article shows a rare case of *H. heilmannii* s.s. infection in a patient with multiple gastric ulcers.

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Acute phlegmonous gastritis complicated by delayed perforation

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Abstract

Here, we report on a case of acute phlegmonous gastritis (PG) complicated by delayed perforation. A 51-year-old woman presented with severe abdominal pain and septic shock symptoms. A computed tomography scan showed diffuse thickening of the gastric wall and distention with peritoneal fluid. Although we did not find definite evidence of free air on the computed tomography (CT) scan, the patient's clinical condition suggested diffuse peritonitis requiring surgical intervention. Exploratory laparotomy revealed a thickened gastric wall with suppurative intraperitoneal fluid in which *Streptococcus pyogenes* grew. There was no evidence of gastric or duodenal perforation. No further operation was performed at that time. The patient was conservatively treated with antibiotics and proton pump inhibitor, and her condition improved. However, she experienced abdominal and flank pain again on postoperative day 10. CT and esophagogastroduodenoscopy showed a large gastric ulcer with perforation. Unfortunately, although the CT showed further improvement in the thickening of the stomach and the mucosal defect, the patient's condition did not recover until a week later, and an esophagogastroduodenoscopy taken on postoperative day 30 showed suspected gastric submucosal

dissection. We performed total gastrectomy as a second operation, and the patient recovered without major complications. A pathological examination revealed a multifocal ulceration and necrosis from the mucosa to the serosa with perforation.

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Key words: Phlegmonous gastritis; Gastric perforation; *Streptococcus pyogenes*

Core tip: Acute phlegmonous gastritis (PG) is a rare and often fatal condition that is characterized by bacterial infection. Patients with PG can present with abdominal pain, abdominal distension, nausea, vomiting, fever, and signs of infection. Computed tomography is useful in the early diagnosis of PG. However, because of the rarity of this disease, the diagnosis and choice of appropriate treatment is difficult. Here, we report a case of acute PG complicated by delayed perforation during conservative treatment after explorative laparotomy without gastric resection.

Min SY, Kim YH, Park WS. Acute phlegmonous gastritis complicated by delayed perforation. *World J Gastroenterol* 2014; 20(12): 3383-3387 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i12/3383.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i12.3383>

INTRODUCTION

Phlegmonous gastritis (PG) is a rare and often fatal disease that is characterized by bacterial infection of the stomach. Patients present with abdominal pain, nausea, vomiting, fever, and signs of infection. The most common pathogens related to PG are members of the *Streptococcus* species. *Streptococcus* accounts for approximately 68% to 75% of all PG cases^[1,2]. Although the pathogen-

esis is not completely known, predisposing factors, such as mucosal injury, immunocompromise, alcohol use, and a history of gastritis have been hypothesized as being important factors^[2-4]. However, approximately 50% of the patients who develop PG were previously healthy. Because of its rarity, the proper treatment of PG is not precisely known; therefore, treatment decisions are difficult. We present a case of acute PG complicated by delayed perforation resulting from unsuccessful conservative treatment.

CASE REPORT

A previously healthy 51-year-old woman was brought to the emergency room. She presented with severe abdominal pain and vomiting. There was no history of alcoholism or other diseases. One day prior to admission, she vomited and experienced upper abdominal pain after dinner; as a result, she visited another hospital. She was diagnosed with usual gastritis and underwent treatment with an H2 blocker. Although she took a pill, the symptoms worsened. Upon examination, the bowel sounds were hypoactive, the diffuse abdomen was tender to palpation, and there was muscle guarding. Her blood pressure was 70/40 mmHg, her heart rate was 122 beats per minute, and her body temperature 36.2 °C.

The patient's laboratory results upon admission were as follows: white blood cell count (WBC), $2.9 \times 10^3/\mu\text{L}$ (normal, $4-10 \times 10^3/\mu\text{L}$), with 92.6% segmented neutrophils (50%-70%); hemoglobin, 14.3 g/dL (12-16 g/dL); platelet count, $308 \times 10^3/\mu\text{L}$ ($150-400 \times 10^3/\mu\text{L}$); aspartate transaminase, 25 U/L (about 40 U/L); alanine transaminase, 18 U/L (about 40 U/L); amylase, 85 U/L (25-125 U/L); and C-reactive protein, 26.03 mg/dL (about 0.3 mg/dL).

Computed tomography (CT) revealed a diffusely distended and thickened whole stomach with intraperitoneal fluid. Compared with a CT scan performed earlier at another hospital, gastric distention and wall thickening had aggravated remarkably (Figure 1). Although we did not find definite evidence of free air on the CT scan, the patient's clinical condition suggested diffuse peritonitis requiring surgical intervention. Our preoperative diagnosis was peritonitis with septic shock. We could not exclude bowel perforation, including gastric wall perforation. We suspected that the cause of the gastric distension was inflammation caused by a hidden, sealed gastric perforation. Because her situation was emergent, the patient underwent aggressive fluid resuscitation and was administered broad-spectrum antibiotics. She was taken to the operating room for an explorative laparotomy without other examinations, such as diagnostic endoscopy. Upon exploration, the stomach was abnormally edematous and appeared pale. The surface of the stomach was covered with whitish fibrinous exudates. The intraperitoneal cavity was filled with large amounts of turbid, straw-colored fluid. The peritoneal fluid was sampled for microbial culture analysis. There was no evidence of gastric or duo-

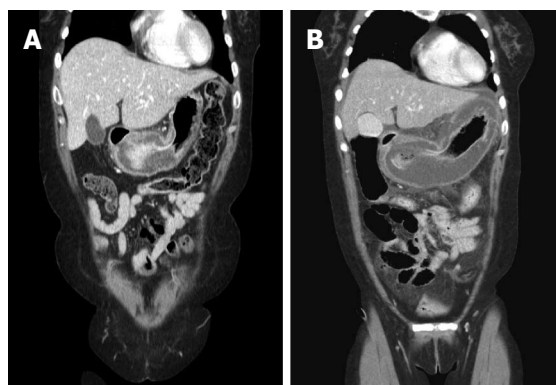


Figure 1 Thickening of the stomach wall was aggravated on abdominal computed tomography. The diameter of the thickened antrum wall increased from 1.7 to 4.0 cm over a 20 h period. A: A computed tomography (CT) reconstruction image taken at another hospital approximately 20 h earlier; B: An initial CT reconstruction image taken at our hospital.

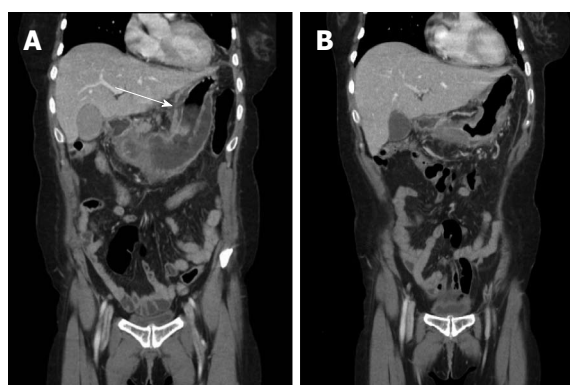


Figure 2 Computed tomography reconstructed images. A: The computed tomography (CT) showed a focal mucosal defect at the lesser curvature side of the body (white arrow), POD 9; B: CT revealed improvement in the state of thickening and fluid collection at the submucosal layer of the gastric body and antrum, POD 29. POD: Postoperative day.

denal perforation. The remaining bowel appeared normal upon inspection. Massive irrigation was performed, and Jackson-Pratt drains were inserted. No further operation was performed at that time. The patient was admitted to the intensive care unit for management.

For the initial 48 h, the patient received intensive support with a cardiac dose of epinephrine, norepinephrine, and fluid to treat septic shock. Although the neutropenia worsened during first 12 postoperative hours, it normalized by postoperative day (POD) 2. After 2 d in the intensive care unit, the patient remained in the general ward for an additional 40 d. She was administered intravenous moxifloxacin for 10 d and piperacillin/tazobactam for 18 d. We prescribed an H2 blocker and administered it with a proton pump inhibitor. *Streptococcus pyogenes* grew from the peritoneal fluid culture. At POD 4, with parenteral nutrition support, the patient started to drink sips of water. After a few days, she was able to consume a liquid diet. Because the patient complained of mild to moderate left flank pain, we performed a CT scan and laboratory tests on POD 9 (Figure 2 and Table 1). The

Table 1 Laboratory values

	Upon admission	Postoperative	POD 2	POD 9	POD 24
WBC (4.0×10^3 - $10.0 \times 10^3/\mu\text{L}$)	2060	520	10290	14340	5980
Hemoglobin (12-16 g/ μL)	13.7	13.9	12.6	13.1	10.1
Platelets (150×10^3 - $350 \times 10^3/\mu\text{L}$)	299	169	94	423	344
Segmented neutrophils (40%-74%)	92		95.2	85	74.8
Prothrombin time (INR)	1.1	1.3	1.1		1.1
AST (about 40 U/L)	25	104	53	57	33
ALT (about 40 U/L)	18	65	45	57	33
Amylase (25-125 U/L)	85	124	29	220	155
CRP (about 0.3 mg/ μL)	26	18.9		6.1	7.5

POD: Postoperative day; WBC: White blood cell; AST: Aspartate transaminase; ALT: Alanine transaminase; CRP: C-reactive protein.

CT scan revealed improved thickening of the gastric wall, but the lesser curvature of the stomach exhibited focal non-enhanced mucosal lesions. This lesion suggested a suspicious focal wall disruption, but no free air was observed. The patient was treated with parenteral nutrition support. Because we provided parenteral nutrition and regular nutritional status assessments were performed, the patient's nutritional status was well maintained. On POD 23, the patient underwent an esophagogastroduodenoscopy (EGD) that revealed a large gastric ulcer and a suspicious perforation site on the high body posterior wall of the stomach (Figure 3). However, because there was no definite evidence of free perforation and the perforated site was covered with fibrotic tissue, we decided to wait until the perforation site healed naturally. Unfortunately, although the CT showed further improvement in the thickening of the stomach and the mucosal defect, the patient's condition did not recover until a week later, and an EGD taken on POD 30 showed suspected gastric submucosal dissection (Figures 2 and 3). We decided to perform a second operation. The gastrectomy revealed that there was no wall dissection, but a thin fibrotic tissue covering a 1.5 cm perforation was found on the high body of the stomach. This perforation site was consistent with prior CT findings. General edema and focal fragile tissue of the wall were also revealed. Because of the gastric wall findings, a total gastrectomy and Roux-en-Y anastomosis were performed.

Gross examination of the specimen revealed a serosal perforated area with diffuse exudative materials. The mucosal surfaces showed a multifocal ulceration and diffuse red-brown discoloration with marked edematous change. Microscopic examination revealed ulceration with necrosis and acute and chronic inflammatory infiltrate from the mucosa to the serosa.

Postoperatively, the patient recovered without complications and was discharged on POD 10 following the second operation. She resumed her regular diet and no longer required antibiotics.

DISCUSSION

Acute phlegmonous gastritis is a rare and potentially fatal condition. It is an acute infection of the stomach wall, submucosa, and muscularis propria by pyogenic bacteria^[5-9]. The etiology of PG is unclear. Predisposing factors, such as alcoholism, mucosal injury, immunocompromise, acquired immune deficiency syndrome, gastric hemorrhage, pregnancy, neutropenia after chemotherapy, and endoscopic procedure have been reported^[6,8,10-13]. Our patient was healthy and had no predisposing factors.

The clinical presentation of PG is nonspecific. The symptoms include epigastric pain, nausea, vomiting, and less often, diarrhea and fever. The usual presentation is severe epigastric pain. Sepsis and multiple organ failure have been frequently reported^[4,14]. The onset of PG is usually rapid^[11]. Moreover, the disease's progression is rapid. PG may follow a rapidly fulminating course, with rapid onset, marked toxemia, and early peripheral circulatory collapse^[15]. In our case, thickening of the gastric wall was rapidly aggravated for only a day.

PG can be diagnosed by endoscopy, endoscopic ultrasound, and CT scan^[15-18]. Although an accurate diagnosis is difficult using examination tools alone, it is possible after appropriate clinical correlation. Because PG is extremely rare and has an atypical presentation, a clinician can misdiagnose it despite performing medical examinations, including CT. Our patient was not administered antibiotics at the other hospitals she first visited; instead, she received a diagnosis of usual gastritis.

PG pathogens are identified from cultures of the peritoneal fluid, gastric aspirates or tissue. Hemolytic streptococcus is the most frequently found organism. Pneumococci, staphylococci, *Proteus vulgaris*, *Escherichia coli*, *Clostridium welchii*, and *Bacteroides subtilis* are also found^[6,15]. PG resulted in the deaths of 41% of the PG patients reviewed, with *Streptococcus* found in 53.3% of the patients who died. Streptococcus was not only the major organism identified but also the most common organism associated with a fatal outcome^[6].

The optimal treatment for PG is controversial. Starr *et al*^[19] reported that the overall mortality rate was 48% after 1945 (when antibiotics became available). Seventy-five percent of the patients died after subtotal gastrectomy, and there was a 50% mortality rate for the patients who were treated with antibiotics only. Kim *et al*^[6] reviewed 36 PG cases from 1975 to 2003. They reported that the mortality rate for patients with surgical resection was 20%, compared with 50% in patients who were treated medically. The mortality rate for localized disease was 10% compared with 54% in patients with diffuse disease. In a review of the 9 PG cases in Korea from 1980 to 2011, Kim *et al*^[20] noted that the mortality rate for patients undergoing surgery was 67%; for patients treated medically, it was 0%. Starr *et al*^[19] recommended treatment options. Subtotal gastrectomy is feasible for the chronic form of PG, and early recognition and prompt intensive supportive management are available for the acute

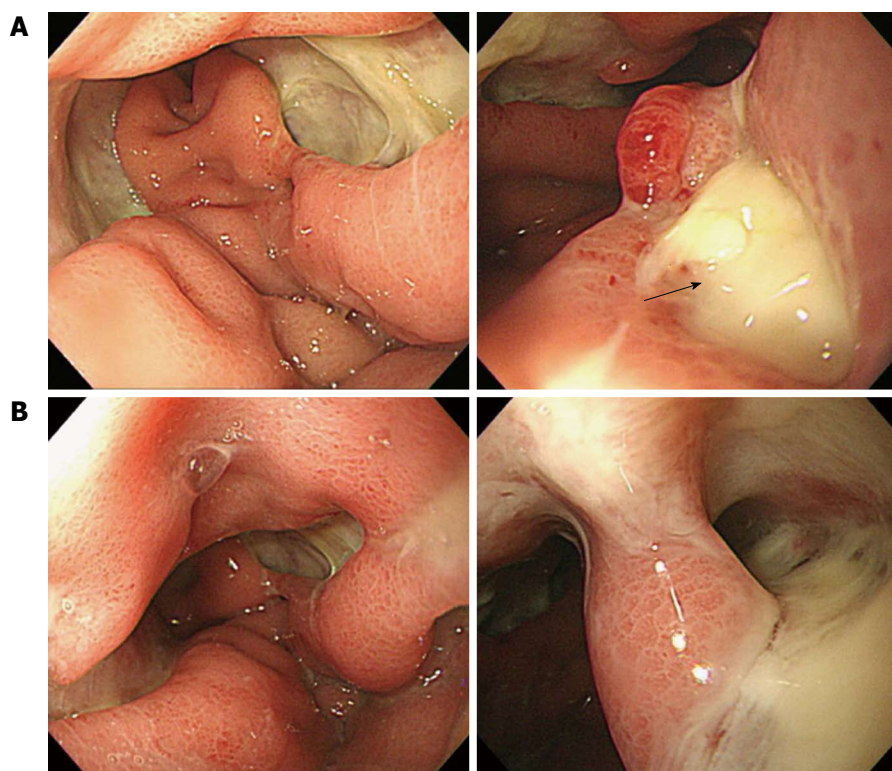


Figure 3 Esophagogastroduodenoscopy. A: A large ulceration was detected (left), and perforation was suspected (right, black arrow), postoperative day 23; B: Submucosal dissection was suspected (left). Magnified image of the area of suspected dissection (right).

form of PG. More recently, total gastrectomy has not been recommended in septic conditions because of high morbidity and mortality, and localized resections are recommended when possible^[4,11]. Resection of the stomach is mainly indicated for complications, including perforation. If operative exploration is undertaken, meticulous evaluation should be performed to exclude a perforation. Endoscopic insufflations should be considered when possible^[1]. The extent and progress of the disease may be a key determination of successful survival and proper treatment choices. As in our case, delayed perforation of the stomach can occur with the diffuse form. The extent of the disease is predictable with CT. CT will reveal a thickened and hypodense gastric wall. Additionally, CT will reveal low-density areas showing peripheral rim enhancement, which is indicative of intramural abscess^[6,21]. Therefore, if diffuse and advanced disease is suspected, resection of the stomach can be considered even when there is no perforation upon exploration. Additionally, if the clinical presentation worsens during conservative management, early evaluation, including CT or EGD, should be performed.

In conclusion, we report a case of acute PG complicated by delayed perforation. PG is a rare and challenging condition. However, proper and successful treatment and survival is possible when it is diagnosed early. We believe that performing early EGD and CT is helpful for both early diagnosis and detecting complications. Additionally, the key to selecting the proper treatment for PG is to precisely predict the extent of the disease.

COMMENTS

Case characteristics

A 51-year-old woman presented with severe abdominal pain and signs of septic shock.

Clinical diagnosis

Diffuse and severe abdominal tenderness and muscle guarding with hypotension.

Differential diagnosis

Gastric perforation, bowel perforation and severe gastritis with infection.

Laboratory diagnosis

WBC, $2.9 \times 10^3/\mu\text{L}$ (normal: $4-10 \times 10^3/\mu\text{L}$), with 92.6% segmented neutrophils (50%-70%); platelet count, $308 \times 10^3/\mu\text{L}$ ($150-400 \times 10^3/\mu\text{L}$); CRP, 26.03 mg/dL (about 0.3 mg/dL); and liver function tests were within the normal limits.

Imaging diagnosis

Computed tomography (CT) showed diffuse thickening of the gastric wall and severe distention with peritoneal fluid.

Pathologic diagnosis

Microscopic examination revealed ulceration with necrosis and acute and chronic inflammatory infiltrate from the mucosa to the serosa.

Treatment

The patient was treated with total gastrectomy because of delayed complications.

Related reports

Acute phlegmonous gastritis is a rare condition. It is an acute infection of the stomach wall, submucosa, and muscularis propria with pyogenic bacteria. The etiology of phlegmonous gastritis (PG) is unclear. Predisposing factors, such as alcoholism, mucosal injury, immunocompromise, acquired immune deficiency syndrome (AIDS), gastric hemorrhage, pregnancy, neutropenia after chemotherapy, and endoscopic procedure have been reported.

Term explanation

Acute phlegmonous gastritis is an acute infection of the stomach wall.

Experiences and lessons

This study reports a case of acute PG complicated by delayed perforation. We believe that performing early esophagogastroduodenoscopy and CT is helpful

for both early diagnosis and detecting complications. Additionally, the precise prediction of the extent of the disease is the key to selecting the proper treatment.

Peer review

Because this is a rare disease, a case report is interesting. Proper evaluation and precise prediction of the extent of the disease is the key for successful treatment and survival.

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Gastric foreign body granuloma caused by an embedded fishbone: A case report

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Abstract

Fishbones are the most commonly ingested foreign bodies that cause gastrointestinal tract penetration. However, fishbones embedded in the gastrointestinal tract that lead to foreign body granulomas that mimic submucosal tumors are rare. Herein, we describe a 56-year-old woman who presented with a 20-day-history of upper abdominal pain. Endoscopy revealed an elevated lesion in the gastric antrum. An abdominal computed tomography scan showed a mass in the gastric antrum and a linear calcified lesion in the mass. An endoscopic ultrasonography examination revealed a 3.9 cm × 2.2 cm, irregular, hypoechoic mass with indistinct margins in the muscularis propria layer. The

patient was initially diagnosed as having a submucosal tumor, and subsequent surgical resection showed that the lesion was a foreign body granuloma caused by an embedded fishbone. Our case indicated that the differential diagnosis of a foreign body granuloma should be considered in cases of elevated lesions in the gastrointestinal tract.

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Key words: Gastric; Foreign body granuloma; Fishbone; Endoscopic ultrasonography; Computed tomography

Core tip: Gastric foreign body granulomas caused by embedded fishbones that mimic submucosal tumors are rare. A gastric intestinal stromal tumor, gastric leiomyoma, and gastric neurofibroma should be considered as differential diagnoses. A computed tomography scan is the most sensitive diagnostic choice, and endoscopic ultrasonography can also be helpful for diagnosis.

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INTRODUCTION

In countries where fish is often consumed, fishbones are the most commonly ingested foreign bodies that become impacted in the upper gastrointestinal tract^[1]. When a foreign body becomes impacted in the upper gastrointestinal tract, 80%-90% of cases resolve spontaneously. However, 10%-20% require nonoperative intervention,

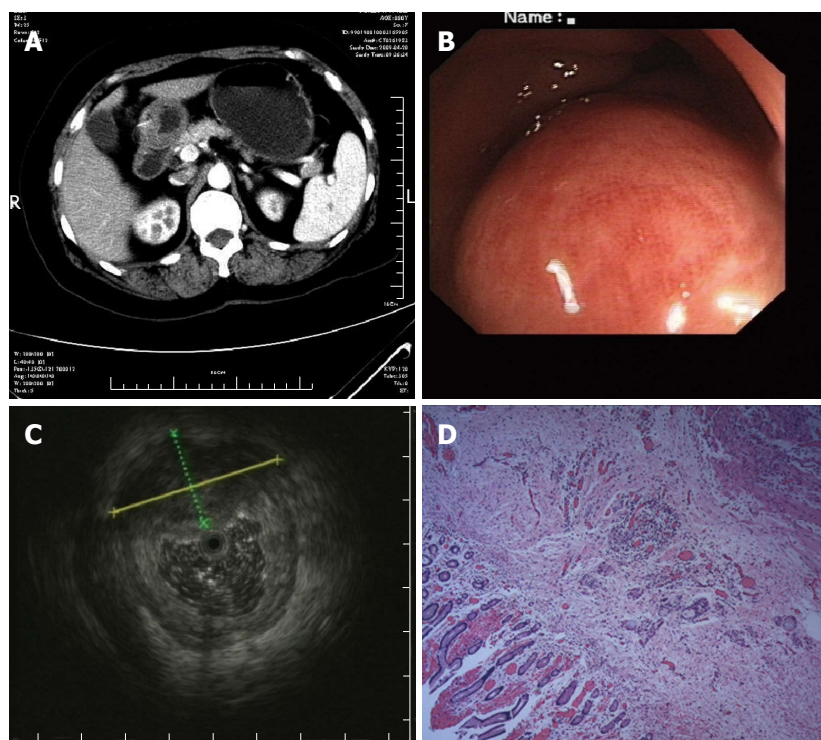


Figure 1 Gastric foreign body granuloma caused by an embedded fishbone. A: A computed tomography scan showed a linear calcified lesion in the stomach; B: Gastroscopy revealed a submucosal tumor in the gastric antrum; C: Endoscopic ultrasonography showed an irregular hypoechoic mass with indistinct margins in the muscularis propria layer; D: Histopathological examination showed chronic and acute inflammatory infiltrates and a foreign body granuloma in the specimen (HE, $\times 100$).

and 1% or fewer require surgery^[2]. However, the chronic lodging of a foreign body in the gastrointestinal tract is rare, especially in the stomach. Here, we report a case of a gastric foreign body granuloma caused by an embedded fishbone that presented with upper abdominal pain. Endoscopy and a computed tomography (CT) scan revealed an elevated lesion in the gastric antrum, which was suspected to be a submucosal gastric tumor. Subsequent surgical resection showed that the lesion was caused by an embedded fishbone.

CASE REPORT

A 56-year-old woman presented with a 20-d-history of upper abdominal pain. The pain had developed gradually over a 20-d-period, with left back radiation. No vomiting or weight loss was reported. She was a farmer without a significant past medical history or a family history of malignancy. Physical examination and routine blood tests, including tumor markers and inflammatory markers, showed no abnormalities. An impression of chronic gastritis was made, and upper gastrointestinal endoscopy was arranged.

The endoscopy revealed an elevated lesion in the gastric antrum, and histopathological examination of a biopsy specimen showed mild chronic inflammatory changes and mild intestinal metaplasia. An abdominal CT scan showed a mass in the gastric antrum and a linear calcified lesion in the mass (Figure 1A). A subsequent endoscopic ultrasonography (EUS) examination revealed a 3.9 cm \times 2.2 cm, irregular, hypoechoic mass with indistinct margins in the muscularis propria layer. EUS also revealed that the mucosal and submucosal layers were mildly thickened, whereas the other layers, the peripheral gastric wall, and the regional lymph nodes were not involved (Figure 1B

and C).

A diagnosis of a suspected submucosal gastric tumor was made. The patient underwent uneventful distal gastrectomy. Histopathological examination of the resected specimen showed that the suspected tumor was a foreign body granuloma surrounding an embedded fishbone (Figure 1D).

DISCUSSION

Fishbones embedded in the stomach wall, forming elevated submucosal lesions that mimic submucosal tumors, are rare. Because the symptoms and signs are vague and nonspecific, the accurate diagnosis of an embedded fishbone remains a challenge for clinicians. Gastric cancer, gastric intestinal stromal tumors, gastric leiomyoma, gastric neurofibroma, and gastric tuberculosis should be considered as differential diagnoses of a gastric foreign body granuloma.

Endoscopy with biopsy is a common method for diagnosing a gastric mucosal lesion. However, regarding gastric submucosal lesions, endoscopic biopsies are usually small and superficial, and it is difficult to obtain a clear diagnosis. In the present case, histopathological examination of an endoscopic biopsy specimen revealed mild chronic inflammatory changes and mild intestinal metaplasia. To improve the endoscopic diagnosis of a submucosal lesion, forceps biopsy, endoscopic mucosal resection, or endoscopic submucosal dissection should be considered.

EUS is a valuable diagnostic tool for submucosal tumors and for the detection of foreign bodies in the gastric wall^[3]. A fishbone presents as a hyperechoic focus on EUS and can appear as a fine needle when the echo is

in parallel with the fishbone. However, in the patient described here, 360-degree EUS was performed, and only a cross-section of the fishbone was observed; this hyperechoic spot was confused with calcification and necrosis. Therefore, longitudinal EUS can have greater sensitivity for the diagnosis of an embedded fishbone than 360-degree EUS.

CT is a useful tool for diagnosing foreign bodies impacted in the gastrointestinal tract. According to previous studies, CT detects impacted fishbones in the esophagus with a sensitivity of 90.9%-100% and a specificity of 100%^[1,4,5]. A fishbone will usually present as a linear calcified lesion on CT images^[6]. In the present case, the CT scan also revealed a linear calcified lesion in the stomach mass.

Endoscopic removal of the fishbone was impossible in this patient because the bone was invisible on the stomach surface. Surgery is usually preferred for patients with embedded fishbones. However, if the embedded fishbone is relatively superior, with an absence of peritonism, endoscopic retrieval might also be possible. In a case of gastric perforation caused by a chicken bone, endoscopic extraction and clipping were successfully performed without peritoneal irritation^[7].

In conclusion, embedded fishbones are rare causes of gastric foreign body granulomas. A CT scan is the most sensitive diagnostic choice, and EUS can also be helpful for diagnosis.

COMMENTS

Case characteristics

A 56-year-old woman presented with a 20-d-history of upper abdominal pain.

Clinical diagnosis

An impression of chronic gastritis was made.

Differential diagnosis

A gastric intestinal stromal tumor, gastric leiomyoma, or gastric neurofibroma.

Laboratory diagnosis

Routine blood tests, including tests for tumor markers and inflammatory markers, showed no abnormalities.

Imaging diagnosis

An abdominal computed tomography scan showed a mass in the gastric antrum and a linear calcified lesion in the mass.

Pathological diagnosis

Histopathological examination of the resected specimen showed that the sus-

pected tumor was a foreign body granuloma surrounding an embedded fishbone.

Treatment

The patient underwent uneventful distal gastrectomy.

Related reports

To our knowledge, no cases of gastric foreign body granulomas caused by embedded fishbones have been reported previously.

Term explanation

A foreign body granuloma occurs due to prolonged exposure to an endogenous or exogenous inflammatory agent. Under these conditions, the mononuclear macrophage system is activated, and foreign bodies steadily accumulate cell infiltrates due to the action of adhesion molecules and chemokines.

Experiences and lessons

The case indicated that the differential diagnosis of a foreign body granuloma should be considered in cases of elevated lesions in the gastrointestinal tract.

Peer review

The authors present a strange case of a foreign body (fishbone) in the gastric wall that simulated a gastric tumor. The case is quite interesting.

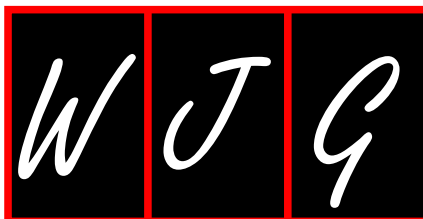
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Journals

English journal article (list all authors and include the PMID where applicable)

- 1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

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- 2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaobua Zazhi* 1999; **7**: 285-287

In press

- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

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- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen

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- 9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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Personal author(s)

- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

- 12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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- 14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

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- 15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/eid/index.htm>

Patent (list all authors)

- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean \pm SD or mean \pm SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as *ν* (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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